Using Next-Generation Sequencing to Develop Molecular Diagnostics for *Pseudoperonospora cubensis*, the Cucurbit Downy Mildew Pathogen

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ABSTRACT

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Advances in next-generation sequencing (NGS) allow for rapid development of genomics resources needed to generate molecular diagnostics assays for infectious agents. NGS approaches are particularly helpful for organisms that cannot be cultured, such as the downy mildew pathogens, a group of biotrophic obligate oomycetes that infect crops of economic importance. Unlike most downy mildew pathogens that are highly host-specific, *Pseudoperonospora cubensis* causes disease on a broad range of crops

belonging to the family *Cucurbitaceae*. In this study, we identified candidate diagnostic markers for *P. cubensis* by comparing NGS data from a diverse panel of *P. cubensis* and *P. humuli* isolates, two very closely related oomycete species. *P. cubensis* isolates from diverse hosts and geographical regions in the United States were selected for sequencing to ensure that candidates were conserved in *P. cubensis* isolates infecting different cucurbit hosts. Genomic regions unique to and conserved in *P. cubensis* isolates were identified through bioinformatics. These candidate regions were then validated using PCR against a larger collection of isolates from *P. cubensis*, *P. humuli*, and other oomycetes. Overall seven diagnostic markers were found to be specific to *P. cubensis*. These markers could be used for pathogen diagnostics on infected tissue, or adapted for monitoring airborne inoculum with real-time PCR and spore traps.

In the past decade, a number of plant pathogens have emerged in the United States causing significant damage in agricultural and natural ecosystems and threatening food security (Fisher et al. 2012). Several recent epidemics have been caused by oomycete pathogens such as the hemibiotroph *Phytophthora infestans* (Fry et al. 2015), the causal agent of late blight of potato and tomato, and obligate plant pathogens within the family *Peronosporaceae* that cause downy mildew (Cohen et al. 2015; Gascuel et al. 2015; Holmes et al. 2015). Downy mildew pathogens include diverse genera such as *Pseudoperonospora* spp. (Gent and Ocampo 2009; Holmes et al. 2015), *Plasmopara* spp. (Gascuel et al. 2015), *Peronospora* spp. (Roberts et al. 2009; Testen et al. 2013), and *Bremia* spp. (Michelmore and Wong 2008) that infect economically important crops and are usually host-specific due to their biotrophic nature. Diagnostic tools for oomycete pathogens range from visual inspection to culturing, serology, and PCR-based tests (Martin et al. 2012). Due to their inability to grow in media and, consequentially, a lack of genomic resources for marker development, downy mildew pathogen diagnostics has mainly been performed by visual inspection of pathogen structures on infected plants (Holmes et al. 2015). However, signs of the pathogen and symptoms of the disease are usually detected when the disease is at advanced stages (>30% severity, Fig. 1) and control strategies, such as fungicide applications, are less effective (Gent et al. 2015; Homa et al. 2014; Ojiambo et al. 2010). In addition, while the main dispersal mechanism of downy mildew pathogens is through airborne sporangia that can travel several miles on wind currents, several downy mildew pathogens are known to be seedborne

(Cohen et al. 2014; Gascuel et al. 2015; Testen et al. 2013). Increasing diagnostic and airborne inoculum monitoring capabilities for downy mildew pathogens could aid in restricting movement of infected plant material (Djalali Farahani-Kofoet et al. 2012) and improving forecasting and alert systems that predict inoculum dispersal (Ojiambo et al. 2015).

Recent studies have developed PCR assays for detection of the downy mildew pathogens of cucurbits (*Pseudoperonospora cubensis*) (Summers et al. 2015), hop (*Pseudoperonospora humuli*) (Gent et al. 2009), basil (*Peronospora belbahrii*) (Djalali Farahani-Kofoet et al. 2012), spinach (*Peronospora effusa*), and beet (*Peronospora schachtii*) (Klosterman et al. 2014) on seed or from air samples to develop alert systems based on primary inoculum levels. PCR primers and markers in these studies were developed from conserved regions such as the internal transcribed spacer (ITS) or mitochondrial genes, resulting in nonspecific amplification of closely related species (Gent et al. 2009; Klosterman et al. 2014). Such assays have limitations for monitoring inoculum in environmental samples since different downy mildew species may be present at the time of sampling due to production of susceptible crops in the same region (Gent et al. 2009; Klosterman et al. 2014). Disease alert systems based on airborne inoculum require species-specific diagnostic assays for timely initiation of disease control measures on a particular crop that is susceptible to its corresponding downy mildew pathogen (Gent et al. 2009; Klosterman et al. 2014).

Next-generation sequencing (NGS) offers the opportunity to generate the genomic resources required to identify species-specific markers through comparative genomics and develop diagnostic assays in downy mildew pathogens as has been done for bacterial plant pathogens (Lang et al. 2010). *P. cubensis* constitutes an interesting case study to test an approach for development of diagnostics that combines generation of limited genomic data with a candidate validation step to identify novel and robust molecular

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diagnostic tools for downy mildew pathogens. *P. cubensis* has an unusually broad host range for a downy mildew pathogen and it can infect diverse and economically important cucurbit crops such as cucumber, cantaloupe, watermelon, squash, and pumpkin, as well as wild cucurbit hosts (Holmes et al. 2015; Runge and Thines 2009; Wallace et al. 2014, 2015). The pathogen is difficult to control since it causes explosive epidemics, may travel long distances via airborne spores, there is no effective host resistance available, and fungicide resistance can quickly develop in pathogen populations (Holmes et al. 2015). Currently, the disease is controlled with intensive fungicide treatments that are initiated once an outbreak is reported in a state or region (Holmes et al. 2015); thus, accurate and timely identification of the pathogen is key for effective disease management. Within *P. cubensis*, there is population structure by host and also geographic region, the latter reflecting host availability in areas such as the southeastern United States (Holmes et al. 2015; Quesada-Ocampo et al. 2012). The closely related species *P. humuli* causes downy mildew on hop (Gent and Ocam 2009), cannot be distinguished consistently from *P. cubensis* based on morphological characters or ITS sequence, and has an overlapping geographical range with *P. cubensis* in several regions (Gent et al. 2009; Mitchell et al. 2011). In this study we aimed to identify *P. cubensis*-specific

diagnostic markers by using a comparative genomics strategy between *P. cubensis* and the closely related species, *P. humuli*.

MATERIALS AND METHODS

Sample preparation for sequencing and candidate validation. Isolates of *P. cubensis* and *P. humuli* used in this study for genome and transcriptome sequencing are listed in Table 1. Isolates were propagated on detached leaves to minimize contamination from other pathogens and insects and to obtain high quality RNA and DNA for sequencing. *P. cubensis* and *P. humuli* isolates were propagated on the host that they were originally isolated from. Detached leaves were placed upside down on moist sterile paper towels inside clear acrylic boxes, spray inoculated with a sporangial suspension of 1 to 3×10^4 sporangia/ml for each isolate, and incubated at 25°C with an 12 h light/dark cycle in a precision plant growth chamber (Thermo Fisher Scientific, Waltham, MA). Sporangia were dislodged from leaves using a Preval sprayer (Preval, Coal City, IL) with sterile water, collected into a tube, and pelleted by centrifugation. Sporangia intended for use in RNA extractions were then suspended in RNALater (Life Technologies,

Fig. 1. Downy mildew symptoms in different cucurbits caused by *Pseudoperonospora cubensis*. **A**, Early infection (<30% severity) on a cucumber leaf; **B**, advanced infection with characteristic angular lesions delimited by leaf veins, and **C**, profuse pathogen sporulation on the underside of the leaf. **D**, Early infection on a cantaloupe leaf; **E**, advanced infection with irregular, necrotic lesions, and **F**, little pathogen sporulation on the underside of the leaf. **G**, Infected watermelon leaf with rounded, necrotic lesions and **H**, little pathogen sporulation on the underside of the leaf.

Grand Island, NY) at a sporangial concentration of 1 mg/μl, while sporangia for DNA extraction were suspended in sterile water. Additional samples used for the diagnostic candidate validation included infected lesions with sporulation or no sporulation collected from field samples (Table 2), mycelia from other oomycetes (Table 2), and uninfected plant tissue grown in the laboratory (Table 3). Samples were stored in microcentrifuge tubes in liquid nitrogen for long-term preservation.

RNA and DNA extraction, library preparation, and sequencing. In preparation for RNA and DNA extraction, 50 μl of sporangial suspension from each isolate was collected in a microcentrifuge tube with 500 μm glass beads (Sigma, St. Louis, MO) and 1 mg of polyvinyl polypyrrolidone (PVPP). Infected lesions, uninfected plant tissue, and other oomycete tissues were each collected into microcentrifuge tubes with three 2.3 mm Zircon beads (Biospec, Bartlesville, OK), 50 mg of 500 μm glass beads (Sigma), and 1 mg of PVPP. All tissues for RNA and DNA extraction were lysed using an OMNI International BeadRuptor (OMNI International, Tulsa, OK). RNA was extracted using the Qiagen Plant RNeasy Kit (Qiagen, Valencia, CA) with an on-column DNase digestion using the Qiagen DNase Digest (Qiagen), both according to manufacturer's instructions. RNA was eluted with 30 to 100 μl of DEPC-treated water and re-precipitated using 1/10 volume of 3 M sodium acetate and two volumes of 96% ethanol, washed with 500 μl of 80% ethanol and resuspended with 30 to 100 μl of DEPC-treated water. DNA extractions were completed using 500 μl of SDS extraction buffer (200 mM Tris-HCl, pH 8, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) followed by one 500-μl phenol and two 500-μl chloroform extractions. DNA was precipitated with 0.5 volume of 100% isopropanol, washed with 500 μl of 80% ethanol, suspended in 100 μl of sterile distilled water, and then reprecipitated with sodium acetate and ethanol as described above, and resuspended in sterile 30 to 100 μl of distilled water. RNA samples for sequencing were quantified using the Qubit RNA HS assay (Life Technologies), DNA samples for sequencing using the Qubit DNA BR assay (Life Technologies), and DNA samples for the diagnostic screen were quantified using a Nanodrop 1000 (Thermo Fisher Scientific). Total RNA was checked for integrity and quality using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). DNA quality assessment was based on gel electrophoresis on a 1% agarose gel containing ethidium bromide at 0.2 μg/ml, followed by detection with a Bio-Rad Geldoc Imager using Quantity One software (Bio-Rad, Hercules, CA).

High quality total RNA and DNA were submitted to the Michigan State University Research Technology Support Facility (MSU-RTSF) for Illumina Truseq RNA (350-bp insert size) and DNA (500-bp insert size) library preparation (Illumina, San Diego, CA). RNA and DNA libraries were barcoded and multiplexed for 50-bp single-end RNA-seq and 100-bp paired-end DNA-seq, respectively. All samples were run on an Illumina HiSeq2500 (Illumina)

and base calling and quality values were determined using the Illumina software as part of the service provided by the MSU-RTSF.

Identification of candidate diagnostic markers and primer design. RNA-seq and DNA-seq reads were analyzed for quality using FastQC (v. 0.10.1) (Andrews 2012). Cutadapt (v. 1.8.1) (Martin 2012) and FASTX-Toolkit (v. 0.0.13) (Hannon 2010) were used to remove adaptors and trim low quality sequences from the data set, respectively. Due to the obligate nature of *P. cubensis* and *P. humuli*, it is likely to find plant contamination in the RNA and DNA samples. Thus, to determine the level of plant contamination in the samples RNA-seq reads were aligned to the *Cucumis sativus* (cucumber) genome (Huang et al. 2009) using Bowtie2 short read aligner (v. 2.1.0) (Langmead et al. 2009). To identify diagnostic candidates, RNA-seq reads from *P. cubensis* and *P. humuli* were aligned to the *P. cubensis* draft genome (Savory et al. 2012a; Savory et al. 2012b) using Bowtie2 short read aligner (v. 2.1.0). The number of aligned reads in each exon of the *P. cubensis* draft genome was quantified using *htseq-count* from HTSeq (v. 0.6.1) (Anders et al. 2015) with default parameters. Exons in the *P. cubensis* draft genome with at least two mapped reads from *P. cubensis* RNA-seq samples and less than two mapped reads from *P. humuli* RNA-seq samples were identified and selected using Perl and Bash programming. Exons present in all *P. cubensis* RNA-seq samples (≥ 2 mapped reads) but absent in all *P. humuli* RNA-seq samples (< 2 mapped reads) were matched to specific genes using the *P. cubensis* draft genome GFF3 file coordinates (Savory et al. 2012a). To further reduce the number of diagnostic candidates to validate with PCR, DNA-seq data were aligned in single end mode to the *P. cubensis* draft genome using Bowtie2 (v. 2.1.0) (Langmead et al. 2009) and mapped reads were quantified using *htseq-count* from HTSeq (v. 0.6.1) (Anders et al. 2015). Candidates were eliminated if two or more DNA-seq reads from *P. humuli* were present in the candidate exon. Approximately 50% of the remaining candidates were validated by PCR against a larger selection of lesions infected with *P. cubensis* or *P. humuli*, other oomycete isolates (Table 2), and host plants (Table 3) to eliminate candidates that occur infrequently, or that are present in more distantly related oomycetes, or in the host (Fig. 2). To determine if candidate diagnostic markers were in putative single copy genes, a self-BLASTP (Altschul et al. 1990) analysis of the *P. cubensis* predicted proteome (Savory et al. 2012a) using an E-value cutoff of $1e^{-10}$ was performed. Candidates were considered single copy if protein sequences had only one match to the proteome. Since several candidate diagnostic markers were annotated as genes of unknown function, a BLASTX analysis against *Phytophthora infestans*, *Phytophthora capsici*, and *Hyaloperonospora arabidopsis* proteins was performed to improve gene functional annotation. The collection of protein sequences were downloaded from the National Center for Biotechnology Information (NCBI) on 3 June 2015 and an E-value cutoff of $1e^{-05}$

TABLE 1. *Pseudoperonospora cubensis* and *P. humuli* isolates used for Illumina 50-bp single-end RNAseq and 100-bp paired-end DNAseq

Pathogen species	Isolate	Host	Location	Year collected	Source
<i>P. cubensis</i>	CA08A1	<i>Cucumis sativus</i> (cucumber)	California	2008	Ojiambo
<i>P. cubensis</i>	SC1982	<i>Cucumis melo</i> (cantaloupe)	South Carolina	1982	Ojiambo
<i>P. cubensis</i>	NC2013C3	<i>Cucurbita maxima</i> (pumpkin)	North Carolina	2013	Ojiambo
<i>P. cubensis</i>	SC2013F2	<i>Cucurbita pepo</i> (acorn squash)	South Carolina	2013	Ojiambo
<i>P. cubensis</i>	NCA11	Cucumber	North Carolina	2012	Ojiambo
<i>P. cubensis</i>	SCD3	<i>Cucurbita moschata</i> (butternut squash)	South Carolina	2012	Ojiambo
<i>P. cubensis</i>	NCWAY2-1	Cucumber	North Carolina	2013	Quesada
<i>P. humuli</i>	NY482CA	<i>Humulus lupulus</i> (hop)	New York	2011	Gent
<i>P. humuli</i>	JP490-5	<i>Humulus japonicus</i> (Japanese hop)	Aomori, Japan	2012	Gent
<i>P. humuli</i>	JP490	Japanese hop	Aomori, Japan	2012	Gent
<i>P. humuli</i>	JP498SA	Japanese hop	Ehoro, Japan	2012	Gent
<i>P. humuli</i>	OR501BA	Hop	Oregon	2013	Gent
<i>P. humuli</i>	OR502AA ^a	Hop	Oregon	2013	Gent
<i>P. humuli</i>	VT503A3	Hop	Virginia	2013	Gent
<i>P. humuli</i>	WI510-1	Hop	Wisconsin	2013	Gent

^a Isolate also used for DNAseq.

was used to define homologous sequences. To further determine that candidates were specific to *P. cubensis*, gene sequences of the diagnostic candidates were used to perform a BLASTN (Altschul et al. 1990) search against the NCBI nucleotide collection (nr/nt) database using default parameters to retain candidates with no significant sequence similarity to other organisms for laboratory validation.

Raw reads were deposited in the Sequence Read Archive at NCBI under accession number SRP063950.

Validation of diagnostic candidates. Fasta sequences of genes containing candidate diagnostic exons and the 200-bp region flanking each gene were selected using *extracseq* from EMBOSS (v. 6.5.7). Primers were designed for *P. cubensis* diagnostic candidates selected for validation within 200-bp 5' and 3' end of the candidate gene using CLC Genomics Workbench (CLCbio, Boston,

MA) and IDT Oligo Analyzer (Integrated DNA Technologies, Coralville, IA). The *P. cubensis* draft genome and the RNA-seq and DNA-seq read alignments were imported into *RNA-seq tools* of the CLC Genomics Workbench to identify primers flanking diagnostic candidates. Primers were designed to produce product sizes between 200 and 1,200 bp with an annealing temperature of 55 to 57°C, and were ordered through IDT (Integrated DNA Technologies). The PCR reactions, completed according to manufacturer's guidelines, contained 1× Promega GoTaq Green Master Mix (Promega, Durham, NC), 10 µM of forward and reverse primers, and 10 µM DNA, and were amplified with a program starting with 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final elongation step of 72°C for 5 min. Products were analyzed by gel electrophoresis on a 2% agarose gel containing ethidium bromide at 0.2 µg/ml, followed by

TABLE 2. *Peronospora belbahrii*, *Pseudoperonospora cubensis*, *Pseudoperonospora humuli*, *Plasmopara obducens*, *Phytophthora capsici*, and *Phytophthora infestans* isolates used for validation of *P. cubensis*-specific diagnostic candidates

Pathogen species or sample	Isolate ^a	Host	Location	Year collected	Source
<i>Peronospora belbahrii</i>	B1	<i>Ocimum basilicum</i> (basil, sweet)	Clayton, North Carolina	2014	Quesada
<i>Peronospora belbahrii</i>	B2	Basil (sweet)	Chapel Hill, North Carolina	2014	Quesada
<i>Peronospora belbahrii</i>	B3	Basil (Genovese)	Roxboro, North Carolina	2014	Quesada
<i>Peronospora belbahrii</i>	B4	Basil (Saim queen thai)	Chapel Hill, North Carolina	2014	Quesada
<i>Peronospora belbahrii</i>	B5	Basil (Genovese)	Chapel Hill, North Carolina	2014	Quesada
<i>P. cubensis</i>	C1	<i>Cucurbita moschata</i> (butternut squash)	South Carolina	2012	Quesada
<i>P. cubensis</i>	C2	<i>Cucumis sativus</i> (cucumber)	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C3	Cucumber	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C4	Cucumber	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C5 (L)	<i>Cucumis melo</i> (cantaloupe)	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C6	<i>Cucurbita pepo</i> (acorn squash)	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C7	<i>Cucurbita maxima</i> (pumpkin)	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C8	Butternut squash	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C9 (L)	<i>Citrullus lanatus</i> (watermelon)	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C10	Butternut squash	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C11	Pumpkin	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C12	Cucumber	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C13 (L)	Cantaloupe	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C14	Cucumber	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C15	Cucumber	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C16	Pumpkin	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C17	Acorn squash	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C18	<i>Cucurbita foetidissima</i> (buffalo gourd)	Waynesville, North Carolina	2014	Quesada
<i>P. cubensis</i>	C19	Buffalo gourd	Waynesville, North Carolina	2014	Quesada
<i>P. cubensis</i>	C20	Butternut squash	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C21	Butternut squash	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C22	Butternut squash	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C23 (L)	Cantaloupe	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C24 (L)	Cantaloupe	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C25 (L)	Cantaloupe	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C26	Cucumber	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C27	Cucumber	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C28	Cucumber	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C29	Cucumber	Michigan	2013	Hausbeck
<i>P. cubensis</i>	C30	Cucumber	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C31	Cucumber	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C32	Cucumber	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C33	Cucumber	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C34	Pumpkin	Kinston, North Carolina	2013	Quesada
<i>P. cubensis</i>	C35	Pumpkin	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	C36	Pumpkin	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C37 (L)	Watermelon	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C38 (L)	Watermelon	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C39 (L)	Watermelon	Cleveland, North Carolina	2013	Quesada
<i>P. cubensis</i>	C40 (L)	Watermelon	Waynesville, North Carolina	2013	Quesada
<i>P. cubensis</i>	1938 (L)	Cucumber	New Jersey	2007	Hausbeck
<i>P. cubensis</i>	1933 (L)	Butternut squash	Puerto Rico	2010	Hausbeck
<i>P. cubensis</i>	1155 (L)	Butternut squash	Italy	2003	Hausbeck
<i>P. cubensis</i>	1923 (L)	Cucumber	Italy	2009	Hausbeck
<i>P. cubensis</i>	1841 (L)	Cucumber	Norway	2009	Hausbeck
<i>P. cubensis</i>	1921 (L)	Cucumber	Germany	2007	Hausbeck

(continued on next page)

^a (L) indicates infected lesion sample with low or no sporulation; (M) indicates mycelia sample; and other samples are lesions.

detection with a Geldoc Imager using Quantity One software (Bio-Rad). Product sizes were estimated by comparison with the Life Technologies 100-bp DNA ladder. Diagnostic candidates with product sizes similar in *P. cubensis* and *P. humuli* samples, as well as any that were amplified in any of the plant hosts or other oomycetes screened were eliminated. Candidates remaining after at least three validation iterations were considered highly specific diagnostic molecular markers for *P. cubensis* (Supplementary File S1).

RESULTS

The approach used in our study successfully identified seven diagnostic markers for *P. cubensis* by using comparative genomics with the closely related species *P. humuli*, a previously published *P. cubensis* draft genome assembly (Savory et al. 2012b), minimal

additional genomic data, and validation of diagnostic candidates with diverse samples (Fig. 2).

Identification of candidate diagnostic markers. A range of 11,942,671 to 29,304,711 million of RNA-seq and 36,599,100 million of DNA-seq reads were obtained and checked for quality (Table 4). In general, only 0.21 to 0.49% of reads were removed from RNA-seq samples and 40% from DNA-seq samples due to low quality and plant contamination (Table 4). High quality RNA-seq and DNA-seq reads aligned to the *P. cubensis* draft genome confirmed that the majority of sequences were from the pathogens (Table 4). The highest and lowest percentages of total aligned RNA-seq reads for *P. cubensis* samples to the *P. cubensis* genome were 95 and 79.9%, respectively. For *P. humuli* samples, the highest and lowest percentage of total aligned RNA-seq reads to the *P. cubensis* draft genome was 92.3 and 66.9%, respectively, and 97.5 and 96% was the highest and lowest total alignment percentage for DNA-seq

TABLE 2. (continued from preceding page)

Pathogen species or sample	Isolate ^a	Host	Location	Year collected	Source
<i>P. cubensis</i>	1924 (L)	Cucumber	Netherlands	2001	Hausbeck
<i>P. cubensis</i>	1925 (L)	Cucumber	Spain	2001	Hausbeck
<i>P. cubensis</i>	1927 (L)	Butternut squash	Spain	2003	Hausbeck
<i>P. humuli</i>	H1	<i>Humulus lupulus</i> (hop)	Mills River, North Carolina	2014	Quesada
<i>P. humuli</i>	H2	Hop	Mills River, North Carolina	2014	Quesada
<i>P. humuli</i>	H3	Hop	Mills River, North Carolina	2014	Quesada
<i>P. humuli</i>	H4	Hop	Oregon	2012	Gent
<i>P. humuli</i>	H5	Hop	New York	2011	Gent
<i>P. humuli</i>	H6	Hop	New York	2011	Gent
<i>P. humuli</i>	H7	Hop	Virginia	2013	Gent
<i>P. humuli</i>	H8	Hop	Oregon	2013	Gent
<i>P. humuli</i>	H9	Hop	Oregon	2013	Gent
<i>P. humuli</i>	H10	Hop	Japan	2012	Gent
<i>P. humuli</i>	H11	Hop	Japan	2012	Gent
<i>P. humuli</i>	H12	Hop	Mills River, North Carolina	2014	Quesada
<i>P. humuli</i>	H13	Hop	Oregon	2006	Gent
<i>P. humuli</i>	H14	Hop	Oregon	2008	Gent
<i>P. humuli</i>	H15	Hop	Washington	2008	Gent
<i>P. humuli</i>	H16	Hop	Oregon	2008	Gent
<i>P. humuli</i>	H17	Hop	New York	2013	Gent
<i>P. humuli</i>	H18	Hop	New York	2013	Gent
<i>P. humuli</i>	H19	Hop	Virginia	2013	Gent
<i>P. humuli</i>	H20	Hop	Czech Republic	2012	Gent
<i>P. humuli</i>	H21	Hop	New York	2011	Gent
<i>P. humuli</i>	H22	Hop	Oregon	2011	Gent
<i>P. humuli</i>	H23	Hop	Mills River, North Carolina	2014	Quesada
<i>P. humuli</i>	H24	Hop	Mills River, North Carolina	2014	Quesada
<i>P. humuli</i>	H25	Hop	New York	2011	Gent
<i>P. humuli</i>	H26	Hop	Oregon	2013	Gent
<i>P. humuli</i>	H27	Hop	Aomori, Japan	2012	Gent
<i>P. humuli</i>	H28	Hop	Oregon	2013	Gent
<i>P. humuli</i>	H29	Hop	Oregon	2007	Gent
<i>P. humuli</i>	H30	Hop	Oregon	2008	Gent
<i>P. humuli</i>	H31	Hop	Washington	2008	Gent
<i>P. humuli</i>	H32	Hop	New York	2013	Gent
<i>P. humuli</i>	H33	Hop	Washington	2006	Gent
<i>P. humuli</i>	H34	Hop	Oregon	2008	Gent
<i>Plasmopara obducens</i>	I1	<i>Impatiens walleriana</i> (impatiens)	Michigan	2014	Hausbeck
<i>Plasmopara obducens</i>	I2	Impatiens	Michigan	2014	Hausbeck
<i>Plasmopara obducens</i>	I3	Impatiens	Michigan	2014	Hausbeck
<i>Phytophthora capsici</i>	P1 (M)	Watermelon	North Carolina	2014	Quesada
<i>Phytophthora capsici</i>	P2 (M)	<i>Capsicum annuum</i> (pepper)	NA	NA	Ristaino
<i>Phytophthora capsici</i>	P3 (M)	<i>Cucurbita pepo</i> (zucchini)	South Carolina	NA	Ristaino
<i>Phytophthora capsici</i>	P4 (M)	Watermelon	South Carolina	NA	Ristaino
<i>Phytophthora capsici</i>	P5 (M)	Pepper	Michigan	NA	Hausbeck
<i>Phytophthora capsici</i>	P6 (M)	Pumpkin	Michigan	NA	Hausbeck
<i>Phytophthora infestans</i>	Pi1 (M)	<i>Solanum tuberosum</i> (potato)	Pennsylvania	2009	Ristaino
<i>Phytophthora infestans</i>	Pi2 (M)	Potato	North Carolina	1994	Ristaino
<i>Phytophthora infestans</i>	Pi3 (M)	<i>Solanum lycopersicum</i> (tomato)	California	1998	Ristaino
<i>Phytophthora infestans</i>	Pi4 (M)	Tomato	Pennsylvania	2009	Ristaino
<i>Phytophthora infestans</i>	Pi5 (M)	Tomato	North Carolina	2009	Ristaino
<i>Phytophthora infestans</i>	Pi6 (M)	Tomato	Virginia	2009	Ristaino
<i>Phytophthora infestans</i>	Pi7 (M)	Potato	North Dakota	2010	Ristaino
<i>Phytophthora infestans</i>	Pi8 (M)	Potato	North Dakota	2009	Ristaino

reads. A small percentage of RNA-seq reads of *P. cubensis* (0.5 to 6.7%) and *P. humuli* (0.5 to 7.5%) isolates aligned to the *C. sativus* reference genome. Similarly, a low percentage (12 to 13%) of DNA-seq reads from a single *P. humuli* isolate aligned to the *C. sativus* reference genome (Table 4). The total *P. cubensis* and *P. humuli* RNA-seq and DNA-seq reads aligned to the *P. cubensis* draft genome and the *C. sativus* reference genome for each sample were sorted in uniquely mapped reads and multiple mapped reads to further assess read quality. A range of 92 to 96% of uniquely mapped reads was observed, confirming the good quality of the RNA-seq reads (Supplementary Fig. S1).

An analysis of exon expression performed on mapped RNA-seq reads indicated that 2,696 *P. cubensis* exons were expressed in all *P. cubensis* isolates, 649 *P. cubensis* exons were expressed in all *P. humuli* isolates, and 19,275 *P. cubensis* exons were expressed in

all sequenced isolates of both species. Moreover, 762 and 1,134 *P. cubensis* exons were found to be not expressed in any *P. cubensis* and *P. humuli* isolates, respectively, and 13,612 *P. cubensis* exons were not expressed in isolates of either species. A total of 70 *P. cubensis* exons were identified from 51 genes that were expressed in all *P. cubensis* isolates and not in any *P. humuli* isolates sequenced (Fig. 3, Supplementary Table S1). Self-BLASTP analyses revealed that 6 of the 51 candidate diagnostic genes were putative single copy genes. Of the 51 identified genes, 29 were annotated as genes or proteins of unknown function. The remaining 22 genes had functional annotations related to virulence (elicitins, elicitors, NPPI and CRN proteins) and signaling, among others. BLAST searches performed against a protein database from *Phytophthora infestans*, *Phytophthora capsici*, and *Hyaloperonospora arabidopsidis* allowed annotation of three more genes. These three genes were putatively identified as involved in functions related to virulence (c27478.0e0), glycolysis (c30239.0e0), and chromatin remodeling (c2555.4e5). Candidate c2163.6e5 had 78% identity (38% query cover and 5e⁻⁸⁷ E value) with a *Phytophthora sojae* hypothetical protein (XM_009528096.1), c31609.0e0 had 93% identity (17% query cover and 2e⁻⁰⁶ E value) with an *Aphanomyces invadans* protein kinase (XM_008871021.1), and c6944.1e0-1 had 89% identity (24% query cover and 8e⁻⁶⁵ E value) with a *Phytophthora parasitica* hypothetical protein (XM_008912304.1).

Validation of diagnostic candidates. An additional filtering step using HTSeq and DNA-seq data from a single *P. humuli* isolate allowed us to eliminate 29 *P. cubensis* exons in 21 genes that were not present in the RNA-seq data of *P. humuli* but were present at the

TABLE 3. Plant hosts used for validation of *Pseudoperonospora cubensis*-specific diagnostic candidates

Host species	Common name	Cultivar
<i>Cucumis sativus</i>	Cucumber	Straight 8
<i>Cucumis melo</i>	Cantaloupe	Hale's Best Jumbo
<i>Cucurbita pepo</i>	Acorn squash	Table Queen
<i>Cucurbita maxima</i>	Pumpkin	Big Max
<i>Cucurbita moschata</i>	Butternut squash	Waltham Butternut
<i>Citrullus lanatus</i>	Watermelon	Mickey Lee
<i>Ocimum basilicum</i>	Basil	Sweet Basil
<i>Humulus lupulus</i>	Hop	Pacific Gem

Fig. 2. Flow diagram describing our approach for identifying candidate diagnostic markers for *Pseudoperonospora cubensis*.

DNA level. Of the remaining 30 genes determined to contain 41 exons unique to *P. cubensis* after bioinformatics analyses, a subset of 16 genes containing 26 exons (~50% of candidates) were chosen for PCR validation with 96 diverse isolates (Table 2). An initial screening with 15 *P. cubensis* and 9 *P. humuli* samples eliminated 15 candidate exons in 8 genes that had a product in *P. humuli* samples indistinguishable in size from the PCR product obtained in *P. cubensis* samples. The final screening for the remaining 11 exons in 8 genes included 40 *P. cubensis*, 34 *P. humuli*, 5 *Peronospora belbahrii*, 3 *Plasmopara obducens*, 6 *Phytophthora capsici*, and 8 *Phytophthora infestans* samples or isolates (Table 2). Eight plant hosts was also included in the validation due to the obligate nature of *P. cubensis* and comprised cucumber, cantaloupe, butternut squash, acorn squash, pumpkin, watermelon, basil, and hop (Table 3). This final screening eliminated 1 exon in 1 gene that had a product in *Plasmopara obducens* samples indistinguishable in size from the PCR product obtained in *P. cubensis* samples. No other candidates presented amplification of a product in other samples that could not be distinguished from *P. cubensis*.

These remaining 10 exons in 7 genes (c10851.1e0, c2555.2e1, c2555.3e7, c3155.4e9, c52.12e13, c572.6e19, and c2183.6e2) are considered highly specific diagnostic molecular markers for

P. cubensis that can be used to distinguish it from its sister species *P. humuli* (Table 5, Fig. 4). Two (c2555.2e1 and c2555.3e7) of these genes were found to be putative single copy genes through self-BLASTP analyses (Table 5). PCR assays with the designed primers and gel electrophoresis of the seven final diagnostic candidates revealed that three candidates (c255.2e1, c2555.3e7, and c10851.1e0) did not produce a PCR product in any *P. humuli* samples tested, while four candidates (c52.12e13, c572.6e19, c2183.6e2, and c3155.4e9) produced a PCR product of different size in some or all of the *P. humuli* samples tested. All diagnostic candidates successfully detected *P. cubensis* in hosts such as watermelon, cantaloupe, and buffalo gourd that typically have lower pathogen sporulation (Table 2, Fig. 5). Interestingly, one diagnostic candidate (c2555.3e7) with unknown function had polymorphism among *P. cubensis* samples tested depending on the host. Samples from infected watermelon, pumpkin, and butternut squash had a larger PCR product than those from cucumber, cantaloupe, and buffalo gourd.

DISCUSSION

NGS allows for development of sequence-based, culture-independent diagnostics of pathogens (Pallen et al. 2010). A recent

TABLE 4. Summary of quality control analysis for *Pseudoperonospora cubensis* and *P. humuli* samples used for Illumina 50-bp single-end (SE) RNAseq and 100-bp paired-end (PE) DNAseq

Pathogen species	Isolate	Molecule	Sequencing mode	Number of total reads	Number of high quality reads	Reads mapped to <i>P. cubensis</i> (%)	Reads mapped to <i>Cucumis sativus</i> (%)
<i>P. cubensis</i>	CA08A1	RNA	SE	23,691,206	23,576,348	92.96	0.49
<i>P. cubensis</i>	SC1982	RNA	SE	22,117,725	22,049,442	93.6	6.24
<i>P. cubensis</i>	NC2013C3	RNA	SE	28,035,867	27,888,243	87.98	0.62
<i>P. cubensis</i>	SC2013F2	RNA	SE	16,956,357	16,886,934	79.89	5.8
<i>P. cubensis</i>	NCA11	RNA	SE	28,101,878	27,962,061	88.33	0.62
<i>P. cubensis</i>	SCD3	RNA	SE	29,304,711	29,218,196	95.08	4.26
<i>P. cubensis</i>	NCWAY2-1	RNA	SE	21,038,800	20,974,369	94.58	6.7
<i>P. humuli</i>	NY482CA	RNA	SE	15,906,769	15,860,532	91.45	7.54
<i>P. humuli</i>	JP490-5	RNA	SE	20,211,068	20,149,698	92.12	4.58
<i>P. humuli</i>	JP490	RNA	SE	11,942,671	11,913,949	66.91	6.45
<i>P. humuli</i>	JP498SA	RNA	SE	26,977,356	26,866,989	90.01	0.54
<i>P. humuli</i>	OR501BA	RNA	SE	14,429,953	14,399,612	88.53	5.55
<i>P. humuli</i>	OR502AA ^a	RNA	SE	26,193,148	26,086,672	92.28	0.51
<i>P. humuli</i>	VT503A3	RNA	SE	14,576,962	14,543,704	91.75	5.69
<i>P. humuli</i>	WI510-1	RNA	SE	29,264,282	29,150,378	88.78	0.68
<i>P. humuli</i>	OR502AA_R1 ^a	DNA	PE	36,599,100	21,778,230	97.46	12.02
<i>P. humuli</i>	OR502AA_R2 ^a	DNA	PE	36,599,100	21,778,230	95.99	13.2

^a Isolate also used for DNAseq paired-end sequencing but reads were aligned to the *P. cubensis* genome as single-end reads (R1 and R2).

Fig. 3. Venn diagram describing results from HTSeq analysis. The number of expressed and not-expressed *Pseudoperonospora cubensis* exons in *P. cubensis* and *P. humuli* isolates sequenced are shown in the Venn diagram.

study used genomics approaches to develop diagnostics for obligate pathogens of humans such as *Parachlamydia acanthamoebae* (Greub et al. 2009). In that study, a draft genome sequence of the emergent pathogen *Parachlamydia acanthamoebae* was obtained and used in combination with proteomics to identify immunogenic proteins and develop a serological diagnostic assay (Greub et al. 2009). However, there have been few examples of genomics-enabled diagnostics development for plant pathogens (De Boer and

TABLE 5. Candidate code, gene identifier, gene functional annotation, primers, and expected product sizes for final *Pseudoperonospora cubensis*-specific diagnostic candidates identified in this study

Candidate code ^a	Gene identifier	Functional annotation	Forward primer	Reverse primer	Product size
c52.12e13	maker-pcu_contig_52-snap-gene-0.12-mRNA-1:exon:13	Crinkler CRN family protein	ATTTCACCTTAGAGTCACGGC	CGGCCATCAACTCGTATCTC	486
c572.6e19	maker-pcu_contig_572-snap-gene-0.6-mRNA-1:exon:19	Gene of unknown function	GGAGACACGACAACAAATGCAG	TGCGCGGCAAGAATTTAGG	854
c2183.6e2	snap_masked-pcu_contig_2183-abinit-gene-0.6-mRNA-1:exon:2	Conserved gene of unknown function	ATGCAGCGGGTATCCTTCC	CACGAAGAAGCAGGAAATGC	448
c2555.2e1 (S)	maker-pcu_contig_2555-snap-gene-0.2-mRNA-1:exon:1	Gene of unknown function	CGCGCAACCTTGAATACCTAC	CACCCGCTCGCAGTAATGAC	831
c2555.3e7 (S)	maker-pcu_contig_2555-snap-gene-0.3-mRNA-1:exon:7	Gene of unknown function	GACGCAACCCAGCATAACAAG	TGCGCTCGAGTATCTTCTACC	787
c3155.4e9	maker-pcu_contig_3155-snap-gene-0.4-mRNA-1:exon:9	Gene of unknown function	TCATCACCATATCCGCTTCTCTG	TGCGTGAATGTGTGCGAC	595
c10851.1e0	maker-pcu_contig_10851-snap-gene-0.1-mRNA-1:exon:0	NPP1	CAGTTCTCGATTTCCTCTCGTC	CTCTACTCACAAGATCTGCCAC	867

^a (S) indicates putative single-copy gene according to self-BLASTP analyses.

Fig. 4. Using next generation sequencing to identify species-specific regions in *Pseudoperonospora cubensis* utilizing comparative genomics with the closely related species *P. humuli*. Seven species-specific regions (c52.12e13, c572.6e19, c2183.6e2, c2555.2e1, c2555.3e7, c3155.4e9, and c10851.1e0) are shown in the figure as columns with the gene functional annotation above each column and the candidate diagnostic code bellow each column. The *P. cubensis* genome panel shows predicted genes in the *P. cubensis* genome that are absent or have missing exons in the *P. humuli* genome. RNA-seq was performed in a diverse panel of *P. cubensis* (CA08A1, SC1982, NC2013C3, SC2013F2, NCA11, NCWAY2-1, and SCD3) and *P. humuli* (OR501BA, NY482CA, VT503A3, JP490-5, JP498SA, WI510-1, and OR502AA) isolates to detect transcripts always present in *P. cubensis* but not present in *P. humuli* by mapping reads to the *P. cubensis* genome using HTSeq. To confirm the absence of the exon or gene at the DNA level in *P. humuli* and develop a PCR-based assay, DNA-seq was performed on *P. humuli* (OR502AA). Primers were designed flanking each gene to detect a size difference in PCR products between *P. cubensis* and *P. humuli* due to the absence of exons or genes in *P. humuli*. This figure was generated using the CLC Genomics Workbench (CLCbio, Boston, MA).

Lopez 2012; Studholme et al. 2011), and most of these studies have been applied to either bacterial (Lang et al. 2010) or viral pathogens (Adams et al. 2009; Studholme et al. 2011). In this study we used a comparative genomics strategy to develop species-specific molecular diagnostics for the obligate oomycete *P. cubensis*, a devastating downy mildew pathogen of cucurbit crops (Savory et al. 2011). A draft *P. cubensis* genome was available for this study; however, this genome is not of finished quality (N50 contig size of 4.0 kbp

(Savory et al. 2012a, b). Nonetheless, our study highlights the usefulness of genomic data even in unfinished format for marker development, especially for obligate pathogens where genomic resources are scarce and obtaining adequate amounts of DNA for laboratory testing may be difficult (Baxter et al. 2010; Links et al. 2011). The RNA-seq data allowed us to identify diagnostic candidates with differential expression between *P. cubensis* and *P. humuli*, but conserved within each species, and thus, robust across isolates

Fig. 5. Gel electrophoresis images showing seven *Pseudoperonospora cubensis* diagnostic candidate PCR products (c52.12e13, c572.6e19, c2183.6e2, c2555.2e1, c2555.3e7, c3155.4e9, and c10851.1e0) for diverse cucurbit downy mildew (C37, C12, C13, C35, C29, C21, C18, and C38) and hop downy mildew samples (H3, H4, H12, H10, H24, H26, H31, and H32). See Table 2 for sample information and Table 5 for primer information. Diagnostic candidate codes corresponding to each gel are indicated on the left side of each gel image.

of these two frequently indistinguishable pathogens. The DNA-seq data allowed confirmation of presence or absence of an exon or a gene at the DNA level in *P. humuli*, since no genomic data are available for this pathogen. Prior knowledge of the genetic structure of *P. cubensis* and *P. humuli* (Mitchell et al. 2011; Quesada-Ocampo et al. 2012) informed our selection of diverse samples for sequencing and likely improved the efficient elimination of unsuitable candidates and increased validation success. The close relatedness between *P. cubensis* and *P. humuli* allowed for rapid identification of lineage-specific regions in *P. cubensis* that can be used for diagnostics, highlighting the importance of resolving phylogenetic relationships for development of robust diagnostics (Choi et al. 2005; Mitchell et al. 2011). Interestingly, several of the candidates identified had no significant sequence similarity to other organisms or informative functional annotation, while other candidates had gene functions related to stress (virulence), as has been observed for lineage-specific genes in plants (Campbell et al. 2007; Lin et al. 2010).

Since the diagnostic candidates were selected based on absence of an exon or a complete gene in *P. humuli*, diagnostic assays can be implemented as conventional PCR-based diagnostics that detect presence of product, product size differences, and or potentially with serological assays. In this study, we pursued validation of diagnostic candidates through PCR methods since such assays can be readily adopted by Plant Disease Clinics as well as extension personnel, while serological methods frequently require adoption and commercialization (De Boer and Lopez 2012). Nonetheless, since some of the diagnostic candidates identified in this study are present in *P. cubensis* but completely absent in *P. humuli* at the DNA level and potentially the protein level, they could be used for future development of serological-based diagnostics once monoclonal antibodies are developed for the *P. cubensis*-specific protein. A clear advantage of serological diagnostics is that it can be incorporated in field-friendly formats that can be used directly by growers, extension agents, and agricultural consultants, as is the case with the *Phytophthora* ImmunoStrip (Adgia, Elkhart, IN) (De Boer and Lopez 2012).

Accurate and timely identification of plant pathogens is the first step toward successful mitigation of crop diseases (De Boer and Lopez 2012). In cucumber, downy mildew symptoms have very characteristic angular leaf spots delimited by leaf veins and profuse sporulation on the underside of the leaf, making diagnosis simple by visual means once the disease has >30% of severity on a leaf (Fig. 1) (Holmes et al. 2015). However, diagnosis during early stages of infection when fungicide applications would be most effective, or in hosts such as watermelon where symptoms are nondescript, can be difficult and delay disease mitigation efforts (Holmes et al. 2015). Recently, *P. cubensis* was also reported to be seed borne as is the case with other downy mildew pathogens such as *Peronospora belbahrii* (Cohen et al. 2014). The contribution of seed borne inoculum to yearly cucurbit downy mildew epidemics remains unclear but movement of infected plant material likely plays a role in *P. cubensis* population changes in the United States (Cohen et al. 2014; Holmes et al. 2015). Molecular diagnostics could be used for detection of early infections or infected seed in combination with appropriate sampling strategies, which would prevent pathogen introduction into new regions (De Boer and Lopez 2012). PCR assays with the seven nuclear genes identified in our study successfully detected *P. cubensis* in infected tissue of all plant hosts tested, including watermelon, which typically yields low quantities of pathogen DNA due to small lesions and poor sporulation of the pathogen on this host (Fig. 5).

Another common method to detect oomycetes is the use of spore traps, which is especially useful for monitoring airborne inoculum of downy mildew pathogens since many species are spread via airborne sporangia (Gent et al. 2009; Granke et al. 2013; Klosterman et al. 2014). In spore traps, pathogen sporangia are impacted on adhesive tape or grease covered rods on hourly, weekly, or daily time intervals that can later be stained and counted under a microscope (Jackson and Bayliss 2011). In U.S. states like

Michigan, a defined threshold of sporangia per week is used to determine when it is prudent to begin fungicide sprays for *P. cubensis* since sporangia concentrations are associated with the timing of downy mildew occurrence (Granke et al. 2014). A drawback of volumetric spore traps that has limited their deployment is the time and labor needed to enumerate sporangia (Granke and Hausbeck 2011), and often, morphological characters such as spore type and size, overlap between closely related organisms (Runge et al. 2012). Limitations of tape-based spore traps have been addressed in other downy mildew pathogens by combining spore trapping with molecular detection and quantification of the pathogen (Gent et al. 2009; Klosterman et al. 2014). Due to its usefulness in identification of fungi and oomycetes, ITS has become a common marker for diagnostics (Schoch et al. 2012), including many downy mildew pathogens (Belbahri et al. 2005; Feng et al. 2014; Gent et al. 2009; Hukkanen et al. 2006; Ioos et al. 2012; Klosterman et al. 2014; Montes-Borrego et al. 2010; Mota et al. 2011; Testen et al. 2013; Valsesia et al. 2005; Zipper et al. 2009). However, in closely related species there may be insufficient variability in the ITS (Gent et al. 2009; Klosterman et al. 2014), or copy number variation (Belbahri et al. 2008; Martin 1990) for useful delineation. Other assays utilizing locked nucleic acid probes and high resolution melt curve analysis can partially overcome this limitation, but add expense to the diagnostics (Summers et al. 2015). Two of the seven diagnostic markers identified in our study are putative single-copy markers (c2555.2e1 and c2555.3e7), making them attractive candidates for developing real-time PCR assays for *P. cubensis* inoculum monitoring efforts. Candidates c2555.2e1 and c2555.3e7 were also two of the three candidates that had no amplification in any *P. humuli* samples tested under the PCR conditions and primers designed in this study (Fig. 5).

Beyond the traditional use of plant disease diagnostics for agricultural decision-making, the need for rapid diagnostics has dramatically increased due to globalization of agricultural products (De Boer and Lopez 2012). Losses due to invasive plant pathogens could be reduced if improved disease detection methods were available (De Boer and Lopez 2012). For obligate parasites, such as downy mildew pathogens, development and validation of molecular assays may be complicated by lack of available genetic material, minimal sequence data to identify diagnostic markers, and unresolved phylogenetic status within the different genera (Thines et al. 2009). NGS and bioinformatics approaches allowed us to develop diagnostic markers that can differentiate *P. cubensis* from *P. humuli*. The approach detailed in this study also may also be amenable to identifying strains or pathotypes of *P. cubensis*. Similar approaches can also be applied to other plant pathogens with scarce genomic resources for rapid development of diagnostic markers.

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