Genome Sequencing and Transcriptome Analysis of the Hop Downy Mildew Pathogen *Pseudoperonospora humuli* Reveal Species-Specific Genes for Molecular Detection

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ABSTRACT

Pseudoperonospora humuli is an obligate oomycete pathogen of hop (*Humulus lupulus*) that causes downy mildew, an important disease in most production regions in the Northern Hemisphere. The pathogen can cause a systemic infection in hop, overwinter in the root system, and infect propagation material. Substantial yield loss may occur owing to *P. humuli* infection of strobiles (seed cones), shoots, and cone-bearing branches. Fungicide application and cultural practices are the primary methods to manage hop downy mildew. However, effective, sustainable, and cost-effective management of downy mildew can be improved by developing early detection systems to inform on disease risk and timely fungicide application. However, no species-specific diagnostic assays or genomic resources are available for *P. humuli*. The genome of the *P. humuli* OR502AA isolate was partially sequenced using Illumina technology and assembled with ABySS. The assembly had a minimum scaffold length of 500 bp and an N50 (median scaffold length of the

assembled genome) of 19.2 kbp. A total number of 18,656 genes were identified using MAKER standard gene predictions. Additionally, transcriptome assemblies were generated using RNA-seq and Trinity for seven additional *P. humuli* isolates. Bioinformatics analyses of next generation sequencing reads of *P. humuli* and *P. cubensis* (a closely related sister species) identified 242 candidate species-specific *P. humuli* genes that could be used as diagnostic molecular markers. These candidate genes were validated using polymerase chain reaction against a diverse collection of isolates from *P. humuli*, *P. cubensis*, and other oomycetes. Overall, four diagnostic markers were found to be uniquely present in *P. humuli*. These candidate markers identified through comparative genomics can be used for pathogen diagnostics in propagation material, such as rhizomes and vegetative cuttings, or adapted for biosurveillance of airborne sporangia, an important source of inoculum in hop downy mildew epidemics.

Hop (*Humulus lupulus*) is an herbaceous and dioecious perennial crop mainly grown to produce beer and ale (Natsume et al. 2015; Neve 1991). During spring, shoots emerge from rhizomes or crowns and grow along a vertical trellis system (Chee et al. 2006; Neve 1991). Flowering begins in the summer on lateral branches, and female plants produce cones that provide the bitterness and hop aroma to beer and ale. This is owing to lupulin glands in cones that produce resins and essential oils, such as alpha and beta acids (Natsume et al. 2015). In the United States, most of the hop acreage is in Washington, Oregon, and Idaho, which comprise ~23% of the world's total acreage (MacKinnon 2004). Hop downy mildew, caused

by *Pseudoperonospora humuli*, is the number one threat to hop production in wet production regions owing to widespread fungicide resistance in the pathogen populations and lack of disease resistance in most hop cultivars (Coley-Smith 1964; Gent and Ocampo 2009; Gent et al. 2008). This biotrophic oomycete can infect hop crowns systemically, which allows overwintering as mycelia and results in infected shoots in the following spring referred to as basal spikes (Coley-Smith 1962). The pathogen can be disseminated in systemically infected planting material: both rhizomes and plants produced from softwood cuttings (Royle and Kremheller 1981). Sporangio-phores bearing sporangia are produced on infected tissue and spread via wind and water splashing to new foliage and cones when environmental conditions are favorable (Gent and Ocampo 2009). Oospores, thick-walled resting spores produced in infected tissue, may play a role in overwintering, but their importance in epidemic development is believed to be minor because of their low rates of germination (Coley-Smith 1962; Gent et al. 2017).

Hop is just one of many crops severely affected by downy mildew diseases. Several downy mildew pathogens have emerged or reemerged and caused devastating damage in food and ornamental crops, such as cucumber (*Pseudoperonospora cubensis*) (Holmes et al. 2015; Ojiambo et al. 2015; Savory et al. 2011), basil (*Peronospora belbahrii*) (Wienandt et al. 2015), rudbeckia (*Plasmopara halstedii*) (Rivera et al. 2016), and impatiens (*Plasmopara obducens*) (Palmateer et al. 2013). Other downy mildew pathogens have been established in the United States for many years, but although

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these pathogens are economically important and taxonomically diverse (Thines 2014; Thines and Choi 2016), they remain largely understudied in part because of the inherent difficulty of working with obligate parasites (Guo et al. 2016). The first downy mildew pathogen genome to be sequenced was *Hyaloperonospora arabidopsidis* (Baxter et al. 2010), the *Arabidopsis thaliana* downy mildew, and since then, other draft and higher-quality genomes have been published for *P. cubensis* (cucurbit downy mildew) (Savory et al. 2012), *Plasmopara halstedii* (sunflower downy mildew) (Sharma et al. 2015), *Peronospora tabacina* (tobacco downy mildew) (Derevina et al. 2015), and *Plasmopara viticola* (grape downy mildew) (Dussert et al. 2016). However, regardless of the expansion of genomics resources for some downy mildew pathogens, connecting genotypes to phenotypes and development of markers for population studies or pathogen diagnostics have not increased at the same rate (Crandall et al. 2018; Wallace and Quesada-Ocampo 2017; Withers et al. 2016).

In the field, downy mildew is typically diagnosed by visual examination of foliar tissue that will display typical disease lesions and in many cases, pathogen sporulation (Fig. 1) (Holmes et al. 2015). Because secondary inoculum from sporulating foliage is critical for downy mildew epidemics, many diagnostic efforts have focused on detection of airborne sporangia by using spore traps

(Gent et al. 2009; Klosterman et al. 2014; Kong et al. 2016; Kunjeti et al. 2016). Diagnostic assays available for downy mildew pathogens are mostly based on detection of the internal transcribed spacer (ITS), which may cross-detect closely related species on a mixed sample (Gent et al. 2009; Klosterman et al. 2014). Nonetheless, many downy mildew pathogens are systemic or seedborne, such as is the case with hop, sunflower, and basil downy mildew, and a combination of serological and molecular diagnostics with robust survey mechanisms is desirable for pathogen detection before planting or before foliar symptoms appear (Roeckel-Drevet et al. 1999; Wyenandt et al. 2015). To address this need, in this study, we used a comparative genomics strategy to identify species-specific markers for detection of *P. humuli*. Specifically, we aimed to (i) generate a draft genome assembly of *P. humuli*; (ii) use the published draft genome of *P. cubensis*, the causal agent of cucurbit downy mildew and sister species of *P. humuli*, to identify *P. humuli* species-specific genes; and (iii) evaluate the putative identified species-specific genes as diagnostic markers for the pathogen.

MATERIALS AND METHODS

Pathogen material, DNA and RNA extraction, library preparation, and sequencing of *P. humuli*. *P. humuli* isolate OR502AA was propagated in detached hop leaves of cultivar Pacific Gem to generate sporangia for DNA extraction and used for genome sequencing as previously described (Withers et al. 2016). Isolate OR502AA and seven other *P. humuli* isolates were also propagated for RNA extraction and sequencing following the same procedure (Table 1). Transcriptome sequencing data from eight *P. humuli* and seven *P. cubensis* isolates published in Withers et al. (2016) were used in this study for the comparative analysis as described below (GenBank BioProject accession number PRJNA293744). For next generation sequencing (NGS), DNA and RNA were extracted from 50 µl of sporangial suspensions of each isolate as described previously (Withers et al. 2016), whereas 2 to 3 mm infected and uninfected leaf tissue segments were used to extract DNA for primer validation using a phenol-chloroform DNA extraction method (Withers et al. 2016), resuspended in 30 to 100 µl of distilled water, and quantified using a Nanodrop 1000 (Thermo Fisher Scientific) according to the manufacturer's instructions. *P. humuli* DNA samples not previously sequenced by Withers et al. (2016) were submitted to The Genomic Science Laboratory at North Carolina State University for library preparation and sequencing in an Illumina NextSeq 500 (Illumina Inc.). Two different insert size DNA libraries (350 and 800 bp) were pair end sequenced at a read length of 100 nucleotides for *P. humuli* OR502AA. The predicted coding genes from *P. cubensis* isolate

Fig. 1. Downy mildew symptoms on hop caused by *Pseudoperonospora humuli*. **A**, Foliar infection with characteristic angular lesions delimited by leaf veins. **B**, Profuse pathogen sporulation on the underside of the leaf. **C**, Cone infection with irregular necrotic lesions. **D**, Chlorotic and stunted shoots or spikes of hop with a systemic infection of *P. humuli*. **E**, Sporangioophores with sporangia on the underside of the leaf at 20× magnification (courtesy of Matthew Bertone).

TABLE 1. *Pseudoperonospora humuli* and *P. cubensis* isolates used for Illumina RNA and DNA sequencing

Pathogen species	Isolate	Host	Location	Year collected	Source	Sequenced molecule	Sequencing type	Read length
<i>P. humuli</i>	NY482CA	<i>Humulus lupulus</i> (hop)	New York	2011	Gent	RNA	Single end	50
<i>P. humuli</i>	JP490-5	<i>Humulus lupulus</i> (hop)	Aomori, Japan	2012	Gent	RNA	Single end	50
<i>P. humuli</i>	JP490	<i>Humulus lupulus</i> (hop)	Aomori, Japan	2012	Gent	RNA	Single end	50
<i>P. humuli</i>	JP498SA	<i>Humulus lupulus</i> (hop)	Ehoro, Japan	2012	Gent	RNA	Single end	50
<i>P. humuli</i>	OR501BA	<i>Humulus lupulus</i> (hop)	Oregon	2013	Gent	RNA	Single end	50
<i>P. humuli</i>	OR502AA	<i>Humulus lupulus</i> (hop)	Oregon	2013	Gent	RNA and DNA	Single end and paired end	50 and 100
<i>P. humuli</i>	VT503A3	<i>Humulus lupulus</i> (hop)	Virginia	2013	Gent	RNA	Single end	50
<i>P. humuli</i>	WI510-1	<i>Humulus lupulus</i> (hop)	Wisconsin	2013	Gent	RNA	Single end	50
<i>P. cubensis</i>	CA08A1	<i>Cucumis sativus</i> (cucumber)	California	2008	Ojiambo	RNA	Single end	50
<i>P. cubensis</i>	SC1982	<i>Cucumis melo</i> (cantaloupe)	South Carolina	1982	Ojiambo	RNA	Single end	50
<i>P. cubensis</i>	NC2013C3	<i>Cucurbita maxima</i> (pumpkin)	North Carolina	2013	Ojiambo	RNA	Single end	50
<i>P. cubensis</i>	SC2013F2	<i>Cucurbita pepo</i> (acorn squash)	South Carolina	2013	Ojiambo	RNA	Single end	50
<i>P. cubensis</i>	NCA11	<i>Cucumis sativus</i> (cucumber)	North Carolina	2012	Ojiambo	RNA	Single end	50
<i>P. cubensis</i>	SCD3	<i>Cucurbita moschata</i> (butternut squash)	South Carolina	2012	Ojiambo	RNA	Single end	50
<i>P. cubensis</i>	NCWAY2-1	<i>Cucumis sativus</i> (cucumber)	North Carolina	2013	Quesada	RNA	Single end	50

MSU1 (GenBank accession number SRX025566.1) reported by Savory et al. (2012) were used for comparative sequence analysis with *P. humuli* OR502AA. Reads generated in this study can be found under BioProject accession number PRJNA354153.

Genome assembly and annotation of the *P. humuli* nuclear genome. HiSeq Illumina 100-bp paired end reads of *P. humuli* OR502AA genomic DNA (all samples were run on an Illumina HiSeq 2500; Illumina Inc.) were analyzed for quality using FastQC version 0.10.1 (Andrews 2012), Cutadapt version 1.8.1 (Martin 2012), and FASTX toolkit version 0.0.13 (Hannon 2010). A preliminary genome assembly of *P. humuli* OR502AA was performed with ABySS version 1.5.2 (Simpson et al. 2009) using a *k-mer* of 71 in paired end library mode. The preliminary assembly was inspected using Blobology for bacterial, plant, and other contamination expected when sequencing an obligate pathogen (Kumar et al. 2013). Results were plotted using the ggplot2 package from R version 3.2.4 (R Core Team 2017; Wickham 2009) (Supplementary Fig. S1). After removing reads associated with contamination microorganisms and host plant, a new ABySS genome assembly was generated with a minimum scaffold length of 500 bp; then, gene structural predictions were generated with the MAKER structural annotation pipeline (Cantarel et al. 2008; Holt and Yandell 2011). Transcriptome assemblies of *P. humuli* OR502AA and seven additional isolates of *P. humuli* were used as transcript evidence (Tables 1 and 2). Assemblies were generated using Trinity (version 2014) with a minimum contig length of 150 bp and the Jaccard clip option (Haas et al. 2013). The completeness of the genome assembly was assessed based on the percentage of coverage of 248 core eukaryotic genes from CEGMA version 2.5 (Parra et al. 2007) (Table 3) and a database of 429 eukaryote groups from BUSCO version 1.1 (Simão et al. 2015). Predicted proteins were functionally annotated using BLASTP searches to NCBI NR database (e value 1e-05; version March 2017), and Uniprot, InterPro, Pfam motifs, and Gene Ontology (GO) terms were assigned by cross-reference using Retrieve ID/Mapping Uniprot tools (The UniProt Consortium 2018) (Supplementary File S1). The *P. humuli* genome length was also estimated through GenomeScope (Vurture et al. 2017) by using DNA-seq read length and *k-mer* profile analyses with Jellyfish (Marçais and Kingsford 2011). The nuclear genome assembly and annotation of *P. humuli* OR502AA can be found under GenBank accession number PRJNA354153 and in figshare (<https://figshare.com/s/5cfeda89bd3d29f3d259> and <https://figshare.com/s/35951fc4569554efdc34>, respectively).

Genome assembly and annotation of the *P. humuli* mitochondrial genome. Illumina raw reads of *P. humuli* isolate OR502AA obtained in this study were used for mitochondrial genome assembly. A de novo assembly was done in SeqMan NGen version 4.1.2 (DNASTAR), and the resulting contigs were screened for mitochondrial genes by BLAST analysis (Altschul et al. 1990) with the mitochondrial genome of *Phytophthora ramorum* (accession

number DQ832718) and *Peronospora tabacina* (accession number KT893455). Mitochondrial contigs were reassembled using the small template assembly option of SeqMan NGen to extend the ends of the contigs and close gaps. Coverage in the assembly was screened manually, and contigs were broken when gaps, low coverage, or inconsistencies were observed and then, reassembled. This iterative process was repeated until the entire mitochondrial genome was assembled. Open reading frames (ORFs) were predicted and annotated with DS Gene version 1.5 (Accelrys) using the universal genetic code with confirmation of gene identities using BLAST analysis against the published mitochondrial genome sequences noted above. Transfer RNA (tRNA) coding regions were placed using tRNAscan-SE version 1.3.1 (Lowe and Eddy 1997). The mitochondrial genome assembly and annotation of *P. humuli* OR502AA can be found under GenBank accession number MH923236 and in figshare at <https://figshare.com/s/61f5dc6091c0271114e4>. Gene order comparisons with other oomycete mitochondrial genomes (*Achlya hypogyna*, KF226724; *Phytophthora ramorum*, DQ832718; *Phytophthora sojae*, DQ832718; *Phytophthora infestans*, NC002387; *Phytophthora nicotianae*, KY85130; *Phytophthora poloncia*, KT946598; *Peronospora tabacina*, KT893455; *Pythium ultimum*, GU138662; *Pythium insidiosum*, AP014838; *P. cubensis*, KT072718; and *Saprolegnia ferax*, AY534144) were done manually by aligning annotated genomes.

Identification of putative RXLR-like and CRN effector proteins. Putative RXLR, RXLR-like, and CRN effector proteins were identified using the effectR and ShinyR packages of R version 3.4.4 and customized Perl scripts (R Core Team 2017). After identification, each effector protein was checked manually for the presence of the corresponding motif. For predictions of signal peptide presence, SignalP version 4.0 in “sensitive” mode was used for all putative effector proteins (Petersen et al. 2011).

Bioinformatics analyses for identification of diagnostic markers. RNA sequencing reads from *P. humuli* and *P. cubensis* were analyzed for quality using FastQC version 0.10.1 (Andrews 2012), Cutadapt version 1.8.1 (Martin 2012), and FASTX toolkit version 0.0.13 (Hannon 2010) as described in Withers et al. (2016). When sequencing an obligate pathogen, it is expected to obtain a mix of host and pathogen sequences as well as a large quantity of phyllosphere microbiome. Thus, to determine the level of host contamination in pathogen sequences, an *H. lupulus* var. *lupulus* (common hop or hop) genome sequence was downloaded from NCBI under GenBank accession number BBPC00000000.1 (Natsume et al. 2015), and RNA-seq reads were aligned using Bowtie2 version 2.1.0 (Langmead and Salzberg 2012) (Table 4). To identify species-specific diagnostic markers, RNA sequencing reads from *P. humuli* and *P. cubensis* were aligned to the *P. humuli* genome assembly generated in this study and the published *P. cubensis* draft genome (Savory et al. 2012) using Bowtie2 version 2.1.0 with default “valid” alignment mode (Langmead and Salzberg 2012). The number of aligned reads for each exon of the *P. humuli* genome assembly was quantified using htseq-count from HTSeq version 0.6.1 (Anders et al. 2015) in “union” mode and normalized using Quantile Normalization with R (Bolstad et al.

TABLE 2. Transcriptome assemblies statistics of *Pseudoperonospora humuli* isolates

Isolate identification	Number of contigs	N50 median contig length (bp)	Longest contig length (bp)	Shortest contig length (bp)
OR502AA	42,862	2,051	10,982	151
JP498SA	31,655	735	6,454	151
W1510-1	26,677	456	4,348	151
OR501BA	19,116	419	3,909	151
VT503A3	22,185	423	5,153	151
NY481J1	42,048	1,390	7,114	151
NY482CA	21,919	473	10,772	151
JP490	15,842	322	3,846	151
JP490-5	27,310	599	10,772	151
NY507570bc	46,600	1,660	9,306	151
NC18668CAS	67,248	2,013	22,752	151
NC18668GAL	108,802	635	8,147	151

TABLE 3. Genome assembly statistics of *Pseudoperonospora humuli*

Number of scaffolds	5,534
Total size of scaffolds (bp)	40,506,691
Longest scaffold (bp)	195,040
Number of scaffolds >1,000	4,511
Number of scaffolds >10,000	1,026
Number of scaffolds >100,000	12
N50 median scaffold length (bp)	19,165
Average length of break (>25 Ns) between contigs in scaffold	111
Scaffold (% N)	0.24
CEGMA complete (%)	97.8
CEGMA partial (%)	98.8

2003; R Core Team 2017). A Perl script was used to identify exons in the *P. humuli* genome with at least two or more quantiles in *P. humuli* RNA samples and less than two quantiles from *P. cubensis* RNA samples. Exons present in all *P. humuli* RNA samples (two or more quantiles) but absent in all *P. cubensis* samples (less than two quantiles) were matched to specific genes using the *P. humuli* genome assembly GFF3 file coordinates (Fig. 2 and Supplementary File S2). A log₂ transformation was used to plot *P. humuli* isolates expression values into a heatmap using “heatmap.2” from the R “gplots” package (R Core Team 2017). To determine if diagnostic markers were in single-copy genes, a self-BLAST (Altschul et al. 1990) analysis of the *P. humuli*-predicted proteins using an e-value cutoff of 1e⁻¹⁰ was performed. Genes were considered single copy if protein sequences had only one match to the proteome. Because several diagnostic markers were annotated as genes of unknown function, a BLASTX analysis (e-value cutoff of 1e⁻⁰⁵) against *Phytophthora infestans*, *Phytophthora capsici*, and *H. arabidopsis* proteins was performed to improve gene functional annotation.

Primer design and validation of candidate diagnostic markers. The species-specific diagnostic markers identified after bioinformatics analyses were validated by polymerase chain reaction (PCR) against a larger collection of lesions infected with *P. humuli* or *P. cubensis*, other oomycete isolates, and host plants. Fast sequences of genes containing diagnostic markers and the 200-bp region flanking each gene were selected using extraseq from EMBOSS version 6.5.7 (Ison et al. 2011). Primers were designed within 200 bp of the 5' and 3' ends of each gene with a diagnostic marker using Geneious 10.2.3 (Biomatters Ltd.). Primers were designed to produce product sizes between 500 and 1,500 bp, with an annealing temperature of 55 to 58°C, and they were ordered through Integrated DNA Technologies or Eton Bioscience, Inc. The PCR reactions, completed according to the manufacturer's guidelines, contained 1× Promega GoTaq Green Master Mix (Promega) and 10 μM forward and reverse primers, and they were amplified with a program starting at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final elongation step of 72°C for 5 min. Products were analyzed by gel electrophoresis on a 2.0% agarose gel containing 0.2 μg/ml ethidium bromide followed by detection with a Bio-Rad Geldoc Imager using Quantity One software (Bio-Rad). Product sizes were estimated by comparison with a 100-bp DNA ladder (Fisher BioReagents exACTGene DNA Ladders; Fisher Scientific). Diagnostic candidates with product sizes similar in *P. humuli* and *P. cubensis* samples as well as any that were amplified

in any of the plant hosts or other oomycetes screened were eliminated. Remaining diagnostic markers after validation were considered highly specific to *P. humuli*.

RESULTS

***P. humuli* genome sequencing, assembly, quality assessment, and functional annotation of predicted genes.** The *P. humuli* isolate OR502AA originally collected in Oregon from the hop cultivar Centennial was sequenced using Illumina (paired end libraries) to 100× coverage. The preliminary genome assembly was inspected for bacterial contamination using Blobology tools (Kumar et al. 2013). As expected for a foliar obligate pathogen, sequenced reads corresponding to prokaryote contaminants, including Xanthomonadales, Pseudomonadales, Sphingobacteriales, Burkholderiales, Flavobacteriales, and Micrococcales, were found. After removing contaminants, a final de novo assembly was performed, resulting in 5,534 scaffolds and spanning 40.6 Mb; this correspond to 53.8 to 50.1% of the predicted genome size, which was estimated to be 75.4 to 80.9 Mb using Feulgen Absorbance Cytophotometry by Voglmayr and Greilhuber (1998). However, genome size estimation with GenomeScope (Vurture et al. 2017) and *k-mer* profile using OR502AA DNA-seq reads (Table 4) resulted in a *P. humuli* genome length of 47.2 Mb (Supplementary Fig. S2) (<http://qb.cshl.edu/genomescope/analysis.php?code=0p5xas2Qiku8bkgO53Nq>).

An N50 (median scaffold length) of 19.2 kb was obtained, with the largest scaffold of 195 kb (Table 3). To further assess the quality of the assembly, BLASTN searches (Altschul et al. 1990) were performed against hop sequences, and the results showed insignificant similarity to the sequences of the host (0.0045% similarity). CEGMA and BUSCO analyses of scaffolds indicated that the gene space of this genome is well represented in the assembly. Although CEGMA analysis indicated 97.8 and 98.9% of complete and partial core eukaryotic gene representations, respectively (Table 3), BUSCO assessment identified only 79% of core genes (Parra et al. 2007; Simão et al. 2015). Of 429 core eukaryotic genes provided by BUSCO, 341 complete single-copy BUSCOs and 66 complete duplicated BUSCOs were found in the assembled genome.

A total of 18,677 predicted genes were obtained from the transcriptomes of eight *P. humuli* isolates (Table 1), summing to a coding space size of 28.1 Mb. Of these, we found evidence of expression for 9,938 (53%) predicted genes in at least one of the isolates. Predicted proteins from gene models were annotated using

TABLE 4. Mapping statistics of RNA-seq reads to the *Pseudoperonospora humuli* genome

Pathogen species	Isolate	Molecule	Sequencing mode	Number of total reads	Number of high-quality reads	Mapped reads to <i>P. humuli</i> (%)	Mapped reads to <i>Humulus lupulus</i> var. <i>lupulus</i> GCA_000831365.1 (%)
<i>P. humuli</i>	NY482CA	RNA	SE	15,906,769	15,860,532	74.24	9.34
<i>P. humuli</i>	JP490-5	RNA	SE	20,211,068	20,149,698	83.06	5.18
<i>P. humuli</i>	JP490	RNA	SE	11,942,671	11,913,949	51.16	9.23
<i>P. humuli</i>	JP498SA	RNA	SE	26,977,356	26,866,989	90.58	0.45
<i>P. humuli</i>	OR501BA	RNA	SE	14,429,953	14,399,612	75.62	6.81
<i>P. humuli</i>	OR502AA	RNA	SE	26,193,148	26,086,672	93.20	0.35
<i>P. humuli</i>	VT503A3	RNA	SE	14,576,962	14,543,704	79.01	6.77
<i>P. humuli</i>	WI510-1	RNA	SE	29,264,282	29,150,378	85.94	0.91
<i>P. humuli</i>	OR502AA_R1 ^a	DNA	PE1	36,599,100	21,778,230	59.40	0.58
<i>P. humuli</i>	OR502AA_R2 ^a	DNA	PE2	36,599,100	21,778,230	55.68	0.6
<i>P. cubensis</i>	CA08A1	RNA	SE	23,691,206	23,576,348	87.10	1.69
<i>P. cubensis</i>	SC1982	RNA	SE	22,117,725	22,049,442	74.88	7.76
<i>P. cubensis</i>	NC2013C3	RNA	SE	28,035,867	27,888,243	81.34	1.92
<i>P. cubensis</i>	SC2013F2	RNA	SE	16,956,357	16,886,934	61.48	8.98
<i>P. cubensis</i>	NCA11	RNA	SE	28,101,878	27,962,061	81.76	0.62
<i>P. cubensis</i>	SCD3	RNA	SE	29,304,711	29,218,196	81.65	4.86
<i>P. cubensis</i>	NCWAY2-1	RNA	SE	21,038,800	20,974,369	61.48	7.16
<i>P. cubensis</i>	MSU1	DNA	SE	57,635,614	1,687,221	49.38	1.72

^a Paired end DNA-seq used for *P. humuli* genome assembly. SE, single end; PE, paired end.

BLASTP search against the NR NCBI database (version March 2017), and 15,037 of these sequences had significant alignments to 10,036 unique proteins. The KAAS tool was used to identify 2,868 metabolic pathways associated with 3,772 predicted gene models (Kanehisa et al. 2016; Moriya et al. 2007). UniProt Knowledge Base (UniProtKB) annotation was successfully assigned to 14,991 predicted gene models by cross-reference using Uniprot (Retrieve/ID mapping) tools (The UniProt Consortium 2016) to identify 10,062 UniProtKB proteins; InterPro and Pfam annotations were assigned to 10,108 and 8,483 predicted gene models, respectively. GO terms were assigned to a total of 7,641 of 18,656 predicted genes. Of these, molecular function ontologies were associated with 5,737 predicted gene models, and functions related to binding (macromolecular complex binding, organic cyclic compound binding, and heterocyclic compound binding) and catalytic activity (hydrolase activity, transferase activity, and catalytic activity acting on protein) were highly represented in these genes (Fig. 3). A total of 3,295 predicted genes were associated with biological processes, and most of these genes were involved in cellular and metabolic processes, such as cellular metabolic process, cellular component organization, organic substance metabolic process, and primary metabolic process among others. Lastly, 4,150 predicted genes models were assigned ontologies from the cellular component module, and proteins

associated with the membrane were highly represented (Supplementary Fig. S3).

Some proteins secreted by oomycetes are essential for a successful host infection (Birch et al. 2006; Bozkurt et al. 2012; Dodds et al. 2009). The major class of cytoplasmic effectors secreted by oomycetes pathogens is the RXLR effectors that contain a conserved RXLR motif. Searching for this motif across the genome sequence makes it feasible to identify candidate RXLR effector genes (Birch et al. 2008, 2009; Jiang et al. 2008; Morgan and Kamoun 2007; Win et al. 2012). A total of 189 putative RXLR and RXLR-like effectors were predicted from the secretome of *P. humuli* isolate OR502AA genome (Supplementary Fig. S4 and Supplementary File S3). A considerably higher number of RXLR motif effectors were identified compared with other RXLR-like effectors, such as QXLR, RXXLR, and QXXLR, in the *P. humuli* genome. In addition to RXLR effectors, CRN (crinkler family candidates) proteins were also identified (Table 5). A total of 49 putative CRN family proteins harboring either one or both LFLAK and HVLV motifs was found. A plethora of other family proteins involved in plant pathogenicity that have been noted in other oomycetes was also determined. The *P. humuli* genome was found to harbor a similar number of pathogenicity proteins compared with other downy mildew and oomycete pathogens (Table 5).

Fig. 2. Flow diagram describing the bioinformatic and laboratory approaches for identifying candidate diagnostic markers for *Pseudoperonospora humuli*. **A.** To generate a reference genome, DNA from *P. humuli* isolate OR502AA was sequenced using the Illumina NextSeq 500 platform, checked for quality, and preprocessed to remove adapters using FastQC and low-quality sequences using cutadapt and FASTX toolkit. *P. humuli* DNA sequences were then assembled using ABySS, and gene predictions were generated with MAKER. **B.** *P. humuli* and *P. cubensis* RNA sequences from eight and seven isolates, respectively, were checked for quality and mapped to the *P. humuli* assembled genome using Bowtie2. Mapped reads for each *P. humuli* and *P. cubensis* isolate were then counted using HTSeq-count, quantile normalized using R, and parsed using a Perl script to select expressed exons in *P. humuli*. **C.** Primers were designed for all candidate molecular marker genes and screened for polymerase chain reaction (PCR) amplification.

Mitochondrial genome. The mitochondrial genome was assembled into a circular molecule of 39,087 bp with a GC content of 22.2% (Fig. 4). A total of 35 known genes (encoding 18 respiratory chain proteins, 16 ribosomal proteins, and an import protein: *ymf16* of the *secY*-independent pathway), the *rnl* and *rns*, and 19 amino acids encoded on 25 tRNA genes were present. In addition, there were four hypothetical proteins (*ymf98*, *ymf99*, *ymf100*, and *ymf101*) in common with other oomycete mitochondrial genomes (Grayburn et al. 2004; Levesque et al. 2010; Martin et al. 2007) and one putative ORF (*orf359*) located between the *rns* and *cox2* genes that is unique to *P. humuli*. BLAST queries of *orf359* to GenBank identified 85% sequence identity with 1,106 bp of *orf306* and flanking sequences from *P. cubensis* (KT072718). The

gene order and position of the putative ORF were the same as observed in *P. cubensis*. The coding regions represent 90.3% of the genome, with 7.7% of this total representing hypothetical coding regions.

With the exception of a putative ORF, the observed gene order in *P. humuli* is the same as *P. cubensis*, but both species are different from *Peronospora tabacina* in that the *rns* is located in the same position but encoded in the opposite direction; this is the same for *Phytophthora* spp. Although the rest of the gene order is the same as *Phytophthora poloncia*, *Phytophthora ramorum*, and *Phytophthora sojae*, there are several inversions in comparison with *Phytophthora infestans* (*atp9-cob* and *atp1-atp6*) and *Phytophthora nicotianae* (*atp9-cob*). The large inverted repeats encompassing the *rnl* and *rns*

Fig. 3. Assignment of Gene Ontology Slim terms for the Molecular Function category to *Pseudoperonospora humuli* predicted gene models. The two most represented functions in the *P. humuli* genome—catalytic activity and binding—are shown in lighter gray (orange) and darker gray (blue) small pie charts, respectively. SAM, S-adenosylmethionine; CoA, coenzyme A.

TABLE 5. Candidate pathogenicity-related genes in *Pseudoperonospora humuli* and other oomycete genomes

Gene family	<i>P. humuli</i> ^a	<i>P. cubensis</i> ^b	<i>Plasmopara halstedii</i> ^c	<i>Hyaloperonospora arabidopsidis</i> ^c	<i>Phytophthora infestans</i> ^c	<i>Pythium ultimum</i> ^c	<i>Albugo laibachii</i> ^c
ATP-binding cassette superfamily	159	69	32	35	127	112	26
Phospholipase	20	22	17	23	43	25	19
Lipase	16	16	24	30	57	36	23
Cysteine protease family	33	20	54	51	64	75	48
Serine protease family	77	65	62	73	106	149	52
Aspartyl protease family	11	10	15	14	19	34	16
Cutinase	5	2	2	2	4	0	3
NPP1_like (necrosis-inducing proteins)	71	67	19	21	27	7	0
Pectate lyase	4	4	3	8	36	15	0
Cytochrome P450	23	17	14	18	30	44	3
Pectinesterase	14	4	5	4	11	0	0
Elicitin	21	12	16	16	45	46	9
RxLR effector family candidates	189 ^d	271	274	134	505	0	49
Crincklers (CRN family candidates)	90 ^d	145	77	20	196	26	3

^a MAKER annotation of *P. humuli* genome.

^b Savory et al. 2012.

^c Sharma et al. 2015.

^d ShinyR packages of R version 3.4.4 (R Core Team 2017), SignalP (Petersen et al. 2011), and manual check.

coding regions observed in *Pythium* spp., *Salrolegnia ferax*, and *A. hypogyna* are not observed in *P. humuli*. Although some gene orders are conserved among these taxa, the gene orders in general are more divergent in *P. humuli* (*secY* to *rps4*).

Identification of species-specific candidate diagnostic markers. To identify species-specific diagnostic markers, the *P. humuli* genome was used as a reference (Fig. 2). Additionally, mapping of high-quality *P. humuli* and *P. cubensis* RNA-seq reads to the *H. lupulus* var. *lupulus* genome (Natsume et al. 2015) resulted in <10% of dataset alignment, whereas only a small percentage of *P. humuli* (0.58 to 0.6%) and *P. cubensis* (1.72%) DNA-seq reads aligned to the hop genome.

Exons expressed in *P. humuli* but not expressed in *P. cubensis* isolates were determined by mapping *P. humuli* and *P. cubensis* RNA-seq reads to the *P. humuli* genome. Alignments of RNA-seq reads of *P. humuli* isolates varied between 74.24 and 93.2% except for isolate JP490, which had an alignment of 51.16%. For *P. cubensis* isolates, RNA-seq alignments were within a range of 61.48 to 87.1%. DNA-seq read alignment of *P. humuli* and *P. cubensis* to the draft *P. humuli* genome was within a range of 49.38 to 59.4% (Fig. 2 and Table 4). Mapped RNA-seq reads were quantified, and a quantile normalization was applied to the data. Candidate genes were selected using an arbitrary criterion of a value of two quantiles or more for expressed exons and less than two quantiles for nonexpressed exons. Thus, 279 exons expressed in all *P. humuli* isolates and nonexpressed in *P. cubensis* isolates were identified. These exons were associated with 242 predicted genes, and examination of their expression across all seven *P. humuli* isolates revealed that putative candidate genes were clustered in three clades, in which 46% were expressed at low levels, 41% were moderately expressed, and only 13% were highly expressed (Fig. 5A). Ontologies for molecular function and biological process categories were associated with 47 and 23 of these predicted genes, respectively. Using BLASTN (query cover 80%), the candidate gene markers were aligned to assembled transcripts from *P. humuli* OR502AA isolate, resulting in 95 of 242 putative gene markers with strong transcript support.

Functions of these 242 candidate genes included RNA binding, ATPase activity, calcium ion binding, DNA binding, and kinase and

endopeptidase activity among others. Of the 23 genes associated with biological process category, 4 were found to be related to defense and pathogenesis. Interestingly, such genes were annotated as hypothetical proteins (Fig. 5B). Additionally, functional annotation revealed that 18 of the candidate gene markers were annotated as virulence related genes (CRN family, secreted RxLRs, elicitors, elicitors, and pectin esterases), 12 were annotated as glycolysis or other metabolic pathway genes, five were annotated as gene regulation-related genes (transcription factors, protein kinase, and phosphatase), three were annotated as coenzyme Q-binding protein (CoQ10 homolog), and two were annotated as ATP-binding cassette superfamily. Of the candidate gene markers, 99 were annotated as genes of unknown function. The remaining candidate markers had functional annotations related to signaling, regulatory, and housekeeping/constitutive genes. To further identify the marker genes with unknown function, BLAST searches performed against protein databases from *Phytophthora infestans*, *Phytophthora capsici*, and *H. arabidopsidis* allowed annotation of three more genes. These three genes were putatively identified as involved in functions related to ATP-binding protein kinase (c124513.0e2), virulence (c124754.15e1), and signaling (c125755.11e2). Additional searches using the Pfam database identified the presence of protein domains in three more candidate marker genes with unknown function: c127532.8e3 as an Mlo protein (PF03094.13) with 7.1×10^{-6} e value, c124006.2e2 as a ribosomal protein with S15 (PF00312.20) with 1.8×10^{-4} e value, and 125745.4e1 as ubiE/COQ5 self-blustransferase family protein (PF01209.16) with 3.1×10^{-12} e value. A self-BLASTX analysis revealed that all but one were putative single-copy genes.

Validation of species-specific diagnostic markers. All 242 candidate genomic markers were chosen for PCR validation of specificity with diverse *P. humuli* isolates. This initial PCR screening with 10 *P. humuli* isolates resulted in a single PCR amplicon for 39 candidate genomic markers that matched with their corresponding predicted amplicon sizes. For the remaining 202 candidate markers, 163 exhibited amplification for only some or none of the 10 *P. humuli* isolates, and 39 candidate markers produced multiple or variable PCR amplicons among the isolates. Therefore, these markers were not pursued in subsequent experiments. The 39 candidate markers that produced single-PCR amplicons were then evaluated against five *P. cubensis* isolates; 10 of these 39 candidate markers produced either no or readily distinguishable PCR amplicons, and they were chosen for the final PCR validation with 59 diverse isolates. The final PCR screen included an additional 15 isolates of *P. humuli*, 23 isolates of *P. cubensis*, five isolates of *Peronospora belbahrii*, two isolates of *Pl. obducens*, six isolates of *Phytophthora* spp., six isolates of *Pythium* spp., and two host plants (cucumber and hop). After the final screening, 4 of 10 candidate gene markers (c125015.3e1, c127446.1e1, c127233.5e3, and c126365.1e5) exhibited a single distinguishable PCR amplicon in all *P. humuli* isolates compared with other isolates and samples tested (Figs. 6 and 7 and Table 6). These four candidate genomic regions were, therefore, considered highly specific molecular markers for *P. humuli*.

All four of these genes were putative single-copy genes according to self-BLASTX against the MAKER standard predicted proteins of *P. humuli*. Three (c127233.5e3, c127446.1e1, and c126365.1e5) of these four genes were found to be of nuclear origin with unknown functions, whereas one (c125015.3e1) was annotated as a homolog of COQ10, a coenzyme Q-binding protein, which is typically a mitochondrial gene. However, additional BLAST queries of c125015.3e1 to GenBank did not identify significant sequence similarity with any known oomycete mitochondrial genes. In addition, c125015.3e1 was not observed in the assembled mitochondrial genome. Except for *P. humuli* isolates, none of the samples tested were found to produce any PCR amplicons for the c125015.3e1 gene, whereas only some *P. cubensis* isolates produced PCR products of different size for the other three marker

Fig. 4. Circular map of the mitochondrial genome of isolate OR502AA of *Pseudoperonospora humuli*.

genes. None of the four candidate genomic makers exhibited any size polymorphism among the *P. humuli* samples tested.

DISCUSSION

The assembled draft genome of *P. humuli* has a size of 40.6 Mb and reflects a partial assembly smaller than those published for other downy mildew pathogens, such as *Hy. arabidopsidis* (78.9 Mb) and *Pl. viticola* (101.3 Mb) (Sharma et al. 2015; Yin et al. 2017). Previous studies have estimated the *P. humuli* genome size to be between 75 and 80 Mb (Voglmayr and Greilhuber 1998). Nonetheless, a quick statistical analysis of DNA-seq reads *k-mer* profiles with GenomeScope (Vurture et al. 2017) provided a *P. humuli* OR502AA genome length of 47.2 Mb, which correlates better to the assembled genome size estimate than the previous estimates. Provided that *P. humuli* has a diploid genome and that OR502AA DNA-seq reads coverage is 100× (GenomeScope requires a diploid genome and a minimum coverage of 25×), it is conceivable that the GenomeScope estimated length of the *P. humuli* genome is more precise than the length initially estimated by Voglmayr and Greilhuber (1998). The CEGMA analyses indicated 97.8 and 98.9% of complete and partial core eukaryotic gene representations, respectively, whereas <80% of BUSCO core genes were identified in our *P. humuli* OR502AA genome assembly, likely reflecting an incomplete nature of the assembly. Regardless, such difference in genome estimation warrants a more complete genome assembly, preferably using third generation sequencing technologies, such as single-molecule, real-time sequencing technique(s).

Elimination of bacterial contaminant DNA data using Blobology tools likely resulted in a more fragmented assembly but of higher quality with a lower number of scaffolds and higher N50 than that of the *P. cubensis* draft genome (Savory et al. 2012). Additionally, the high percentages from CEGMA and BUSCO reports suggest that the gene space regions from this partial genome assembly can be used in gene-calling pipelines as reported in other oomycetes, such as *Albugo laibachii* (Kemen et al. 2011). Although partial, this is the first reported genome assembly of *P. humuli*, an important pathogen of hop, and it will serve as a draft genome assembly until a more complete genome assembly is generated. The release of the *P. humuli* draft genome is a valuable addition to the oomycete genome resources, especially of downy mildew pathogens, that will provide important insights about the biotrophic lifestyle, and it will serve as a resource for studying coevolution of host and obligate pathogens and speciation owing to host adaptation and reproductive isolation.

UniProtKB, InterPro, and Pfam annotations revealed a plethora of pathogenicity-related proteins, which are described in Table 5. The number of putative pathogenicity-associated proteins identified in the *P. humuli* genome was broadly similar to that identified in other oomycete pathogens. Although the total number of candidate RXLR-type effectors identified in *P. humuli* is less than that reported in *P. cubensis* (Savory et al. 2012) and *Pl. halstedii* (Sharma et al. 2015), it is higher than in *Hy. arabidopsidis* (Table 5). Additionally, most of the variants of RXLR motifs, such as QXLR identified in *P. humuli*, lack the EER motif (Fig. 4) unlike its sister species *P. cubensis*, where all 47 QXLR motifs were noted to have EER motifs (Tian et al. 2011). However, although most of the putative RXLR effectors (93 of

Fig. 5. Gene Ontology and heatmap of candidate molecular markers genes of *Pseudoperonospora humuli*. **A**, Expression values (read counts) were quantile normalized with R and transformed to log₂. Clustering of expression values for the 242 molecular markers candidate genes indicates that most of these genes (46%) are low expressed, that 41% are medium expressed, and that 13% are highly expressed in the transcriptomes across all seven different *P. humuli* isolates. **B**, Gene Ontology Slim functional annotation was assigned to a subset of molecular marker candidate genes—23 genes were associated with different biological processes, and 47 genes were associated with different molecular functions. mRNA, messenger RNA; rRNA, ribosomal RNA. SSU, small subunit; ETS, external transcribed spacer.

101) identified in *P. humuli* are RXLR-EER type, only 34 of 274 of RXLR-like effectors have been found in *Pl. halstedii* to have EER. This raises an interesting question about the conserved role of RXLR-like motifs in conjunction with EER for protein translocation in different oomycete species and is suggestive of involvement of additional proteins (Sharma et al. 2015). It should also be noted that the partial nature of the assembled genome of *P. humuli* could contribute to the identification of a lower number of effector genes compared with its sister species *P. cubensis*.

Crinkler (CRN) family proteins, which are known to suppress host immunity and belong to an ancient class of cytoplasmic effectors, have been documented in the genomes of all sequenced oomycete pathogens (Sharma et al. 2015). Of 49 CRN-like proteins identified, only four were noted to have both LFLAK and HVLV motifs, and the rest exhibited the presence of either of the two motifs. Surprisingly, 47 of 49 proteins with a CRN signature in *P. humuli* were not predicted to carry a signal sequence for secretion. However, similar absence of signal peptide has also been noted in CRN family proteins in *Pl. halstedii* (Sharma et al. 2015). The role of these putative CRN family proteins in pathogenicity or cellular functions remains to be investigated.

The RNA-seq data used in this study for identifying *P. humuli* species-specific diagnostic markers were checked for quality and preprocessed in our previous study (Withers et al. 2016), thereby ensuring the quality of the reads used. Obtaining good quality

genomic DNA or RNA from obligate pathogens can be challenging because of the possibility of recovering a mixture of host and other phyllosphere microbial DNA/RNA together with target pathogen nucleic acids. As a result, alignment of a very low percentage (<10%) of the RNA-seq reads of *P. humuli* and *P. cubensis* isolates with the hop genome was not unexpected. However, RNA-seq reads of almost all *P. humuli* isolates mapped to the *P. humuli* draft genome to a considerably higher extent (>74%). Because infected hop leaves used to grow isolates cannot be completely sterilized, the presence of contaminating microbial DNA and RNA in the NGS dataset used in this study was anticipated. Presence of contaminating nucleic acid sequences from phyllosphere microorganisms in other obligate oomycetes NGS datasets has also been noted (Burkhardt and Day 2016; Guo et al. 2016; Sharma et al. 2015).

Although strong transcriptional evidence was noted in 95 of 242 candidate gene markers, none of them generated PCR amplicons in all tested *P. humuli* isolates, and some were amplified in *P. cubensis* isolates under the PCR conditions used. This could be owing to multiple reasons, such as presence/absence of sequence polymorphism in primer annealing sites, indels making the target too large for the amplification conditions used in *P. humuli* isolates, and/or the unfinished quality (N50 contig size of 4.0 kb) of the *P. cubensis* genome, which allowed false identification of exons as unique in *P. humuli*. One of the advantages of developing markers

Fig. 6. Use of next generation sequencing data to identify species-specific regions in *Pseudoperonospora humuli* utilizing comparative genomics with closely related sister species *P. cubensis*. The *P. humuli* genome panel shows four predicted genes in *P. humuli* that have missing exons in the *P. cubensis* DNA-seq. A diverse panel of RNA-seq data from *P. humuli* and *P. cubensis* isolates (Table 1) was mapped against the *P. humuli* genome to detect transcripts consistently present in *P. humuli* but not in *P. cubensis*. Images were generated using CLC Genomics Workbench (CLCbio).

based on transcriptome evidence is that such markers are associated with functional genes (Fig. 7), which could be used for pathogen detection in combination with tracking a particular phenotype governed by the gene (Wallace and Quesada-Ocampo 2017). A number of candidate genes encoding virulence-related proteins, including RXLR, CRN, and elicitors, were identified as *P. humuli* species specific. Variation or divergence in effector repertoires has been proposed to play an important role in ecological speciation, resulting in host jump or host range expansion (Schulze-Lefert and Panstruga 2011). In plant pathogens, especially in obligate parasites, it is expected that host adaptation, host range expansion, and host jumps are major mechanisms driving speciation (Stukenbrock 2013). Because of high genetic similarities between the two sister species *P. humuli* and *P. cubensis*, additional investigation of these stress-related candidate marker genes could shed light on their role in host adaptation of *P. humuli*. Although none of the effector or virulence-related genes made it to the final four candidate markers for *P. humuli*, c125015.3e1 was consistently amplified in all *P. humuli* isolates but not in any other species' DNA tested. Although our functional annotation pipeline identified c125015.3e1 as a homolog of the mitochondrial CoQ10 protein, additional BLAST analyses did not provide any significant homology between c125015.3e1 and available CoQ10 DNA sequences on the NCBI database. It was, therefore, designated as a gene with unknown function in our final analyses (Table 6). However, CoQ10 is a conserved CoQ-binding protein that shuttles CoQ to the catalytic sites of the respiratory chain (Barros et al. 2005). This makes c125015.3e1 a potential candidate gene for additional investigation of genetic mechanisms that are involved in adaptation of *P. humuli* on hop. Additionally, 99 of 242 candidate marker genes were annotated as genes with unknown function; whether these genes play any role in host specialization of *P. humuli* is unknown. Notably, a set of genes of unknown function exhibited strong positive selection in *Mycosphaerella graminicola* infecting wheat and have been speculated to be involved in host specialization (Stukenbrock et al. 2010).

The mitochondrial genome of *P. humuli* is circular and ~39 kb, which is similar in size to what is observed for *P. cubensis* (38.5 kb), *Peronospora tabacina* (43 kb), *Phytophthora* spp. (~38 to 43 kb), and *Pythium ultimum* (37.7 kb with only one arm of the inverted repeat, 59.7 kb total). The mitochondrial genome encodes the same standard suite of genes, including the putative ORFs *yfm16*, *yfm98*, *yfm99*, *yfm100*, and *yfm101*, such as has been reported for the genus *Phytophthora* (Martin et al. 2007) and *Pythium ultimum* (Levesque et al. 2010) as well as *S. ferax* (Grayburn et al. 2004), *A. hypogyna*, and *Thraustotheca clavata* (O'Brien et al. 2014). There is also an additional putative ORF of unknown function encoded between the *rns* and *cox2* genes that shares some level of sequence identity with a putative ORF in the same location as with *P. cubensis* (KT072718). An inverted repeat that is present in some oomycetes (Grayburn et al. 2004;

Hudspeth et al. 1983; Levesque et al. 2010; McNabb et al. 1987; O'Brien et al. 2014) is not observed in *P. humuli*.

In general, the mitochondrial gene order in *P. humuli* is similar to what was observed in other downy mildews and the genus *Phytophthora* with some exceptions. One notable difference, which was also observed in *P. cubensis* (KT072718), was that the *rns* is encoded in the opposite orientation in *P. humuli* in comparison with *Peronospora tabacina* and *Phytophthora* spp. Other than this, the gene order was the same as observed in *Peronospora tabacina* and *P. cubensis*. With the exception of *rns*, the same gene order was also observed in *Phytophthora ramorum* (DQ82718) and *Phytophthora sojae* (DQ82717), but in comparison with *Phytophthora infestans* and other clade 1C species, there were two inversions: *cob-atp9* and *nad3-atp1* (NC002387, AY898627, AY898628, AY894835, HM590418, HM590419, HM590420, and HM590421). Using a similar approach that was used for developing a diagnostic assay for *Bremia lactucae* (Kunjeti et al. 2016), the mitochondrial genome of *P. humuli* is also being used to develop a mitochondrial-based detection assay (M. Ramon, A. Burkhardt, and F. N. Martin, *personal communication*).

In earlier studies, nuclear ITS and mitochondrial *cox2* region have been utilized for developing *P. humuli* detection markers (Gent et al. 2009; Summers et al. 2015). However, these markers either could not differentiate between *P. humuli* and *P. cubensis* (Gent et al. 2009) or were based on a single-nucleotide polymorphism (SNP) (Summers et al. 2015). Nonetheless, the detection limit of SNP-based real-time PCR assays may exhibit high variability (Psifidi et al. 2011). NGS technologies have been used in several studies, where total DNA and/or RNA were used for the identification of unique regions or sequence variation in nuclear or other cell organelle DNA for diagnostic purposes (De Boer and Lopez 2012; Studholme et al. 2011; Withers et al. 2016). To our knowledge, this is the first report of species-specific *P. humuli* exons or genes that can be used for identification and diagnostics from its closely related sister species *P. cubensis*. Although 242 species-specific candidate exons in 241 genes were identified, based on comparative genomics analyses, not all of the markers amplified during PCR validation screen. This could be because of, among other reasons, designing the PCR primers within 200-bp upstream and downstream regions of each gene's start codon, which might differ among the *P. humuli* isolates and therefore, fail to amplify. Similarly, the 39 candidate genes eliminated after the first round of PCR screening owing to either absence or generation of multiple and/or differential amplicons could be because of PCR primer cross-reaction, amplification stringency, or polymorphism. Allelic polymorphism has been detected in *P. cubensis* (Withers et al. 2016) using similar techniques of candidate marker development; additional investigation is required to confirm any such allelic polymorphism in *P. humuli*.

TABLE 6. Candidate code, exon identification, gene name, gene functional annotation, primers, and expected polymerase chain reaction amplicon sizes for final *Pseudoperonospora humuli* species-specific candidate genomic markers

Candidate code	Exon identification ^a	Gene name	Gene functional annotation	Primer	Sequence	Expected product size
c125015.3e1	maker-contig_125015-augustus-gene-0.3-mRNA-1:1	Phum_OR502AA_v1_g_09538	Gene of unknown function	Forward Reverse	GCTTCCTTTGCGGTACATA TTGTATGAACCTTTGATGCATGT	639
c127446.1e1	maker-contig_127446-snap-gene-0.1-mRNA-1:1	Phum_OR502AA_v1_g_00649	Gene of unknown function	Forward Reverse	AGCCCTAGGAAATTTTCATCTTT ATTGCTTCTTGGTCATTGAAA	632
c127233.5e3	maker-contig_127233-snap-gene-0.5-mRNA-1:3	Phum_OR502AA_v1_g_12093	Gene of unknown function	Forward Reverse	ATACCAAGCACAAAGAACAATA CGTAGTCGCAGTAGATCAC	860
c126365.1e5	maker-contig_126365-snap-gene-0.1-mRNA-1:5	Phum_OR502AA_v1_g_16807	Conserved gene of unknown function	Forward Reverse	ATTCCAATACAAGCACTTCTCT GATCTGCAGTGATTCCATAATG	1,425

^a mRNA, messenger RNA.

Methods utilizing a combination of PCR-based technologies and spore traps have been developed for species-specific detection of downy mildew pathogens of spinach (*Peronospora effusa*), beet (*Peronospora schachtii*) (Klosterman et al. 2014), lettuce (*B. lactucae*) (Kunjeti et al. 2016), cucumber (*P. cubensis*), and hop (*P. humuli*) (Gent et al. 2009; Summers et al. 2015). Sensitivity and specificity of detection to the target organism are of paramount importance for developing such a detection method to avoid cross-detection. Species-specific exons or genes of *P. humuli* identified and PCR validated in this study can be implemented when developing similar DNA-based detection methods, such as quantitative real-time PCR and loop-mediated isothermal amplification. Although hop production in the western United States does not overlap with cucurbit production (Gent et al. 2009), this is not always the case in the East Coast or Midwest regions of the United States, such as in the western part of North Carolina, where cucurbits and hop are grown in the same production regions (Rahman et al. 2017). Because of the systemic growth of *P. humuli* (Gent et al. 2009) in hop and possible presence of aerial sporangia of *P. cubensis* from infected cucurbit plants in the same area, markers identified in this study could be extremely valuable for development of a robust pathogen detection system in symptomless tissues to verify that planting materials are free of *P. humuli*, which is an important source of initial inoculum (Skotland and Johnson 1983). The markers could also be

valuable for early detection of aerial *P. humuli* sporangia for timing fungicide applications and improving management of hop downy mildew (Gent et al. 2009).

Conclusion. Whole-genome sequencing and comparative genomics approaches to identify *P. cubensis* species-specific markers have been successfully applied in a previous study (Withers et al. 2016), and similar approaches in combination with sequencing of the *P. humuli* draft genome allowed us to identify species-specific gene markers for *P. humuli*. Currently, there is a growing demand for high-quality hop in the United States and other parts of the world, because beer has become the most widely consumed alcoholic beverage in the world (Natsume et al. 2015). With an increase in production and resurrection of hop growing in the Eastern and Midwestern United States, hop downy mildew has the potential to become more widespread. Additionally, because of increasing atmospheric moisture content in the Eastern and Midwestern United States (Hill 2018), management of any in-season downy mildew on hop will pose significant challenges and therefore, underlines the necessity of rapid diagnosis and more sustainable disease management strategies. Because detection of *P. humuli*-infected plant materials as well as biosurveillance of airborne inoculum require robust genetic markers for accurate diagnosis, the species-specific candidate genomic markers for *P. humuli* identified in this study will pave the way for the development of rapid molecular diagnostic techniques.

Fig. 7. Gel electrophoresis images of polymerase chain reaction amplicons of four *Pseudoperonospora humuli* candidate diagnostic molecular marker genes (c125015.3e1, c127446.1e1, c127233.5e3, and c126365.1e5) for diverse hop downy mildew and cucurbit downy mildew samples. Table 6 shows primer information, and Supplementary Table S1 shows isolate information.

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