

Symposium contribution/Contribution à un symposium

# Molecular approaches for biosurveillance of the cucurbit downy mildew pathogen, *Pseudoperonospora cubensis*

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**Abstract:** Globalization has allowed for rapid movement of plant pathogens that threaten food security. Successful disease management largely depends on timely and accurate detection of plant pathogens causing epidemics. Thus, biosurveillance of epidemic plant pathogens such as *Pseudoperonospora cubensis*, the causal agent of cucurbit downy mildew, is becoming a priority to prevent disease outbreaks and deploy successful control efforts. Next Generation Sequencing (NGS) facilitates rapid development of genomics resources needed to generate molecular diagnostics assays for *P. cubensis*. Having information regarding the presence or absence of the pathogen, amount of inoculum, crop risk, time to initiate fungicide applications, and effective fungicides to apply would significantly contribute to reducing losses to cucurbit downy mildew. In this article, we discuss approaches to identify unique loci for rapid molecular diagnostics using genomic data, to develop molecular diagnostic tools that discriminate economically important pathogen alleles (i.e. mating type and fungicide resistance), and how to use molecular diagnostics with current and future spore trap strategies for biosurveillance purposes of important downy mildew pathogens. The combined use of these technologies within the already existent disease management framework has the potential to improve disease control.

Keywords: biosurveillance, diagnostics, downy mildew, genomics, next generation sequencing, oomycetes

**Résumé:** La mondialisation a accéléré la dissémination des agents infectieux des plantes qui menacent la sécurité alimentaire. Une gestion efficace des maladies dépend largement de la détection opportune et précise des agents pathogènes responsables des épidémies. Ainsi, la biosurveillance des agents pathogènes des plantes qui causent des épidémies, comme *Pseudoperonospora cubensis*, l'agent causal du mildiou des cucurbitacées, devient une priorité en ce qui a trait à la prévention de flambées de maladies et au déploiement de mesures de lutte efficaces. Le séquençage de nouvelle génération (SNG) facilite l'essor rapide des ressources génomiques requises pour développer des tests de diagnostic moléculaire propres à *P. cubensis*. Le fait d'être informé de la présence ou de l'absence de l'agent pathogène, de la quantité d'inoculum, du risque pour les cultures, du moment approprié pour procéder aux applications de fongicide et des bons fongicides à utiliser, contribuerait grandement à réduire les pertes causées par le mildiou des cucurbitacées. Dans cet article, nous discutons des approches permettant d'identifier des locus uniques afin de poser rapidement un diagnostic moléculaire basé sur des données génomiques et de développer des outils de diagnostic moléculaire qui distinguent les allèles pathogènes importants sur le plan économique (c.-à-d. type sexuel et résistance aux fongicides). De plus, nous y traitons des façons d'utiliser le diagnostic moléculaire de concert avec des stratégies courantes et futures de piégeage de spores aux fins de la biosurveillance d'importants agents pathogènes causant le mildiou. L'utilisation conjointe de ces technologies dans le cadre actuel de gestion des maladies peut contribuer à améliorer les mesures de lutte contre ces dernières.

Mots clés: Biosurveillance, diagnostics, génomique, mildiou, oomycètes, séquençage de nouvelle génération

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### Introduction

Pseudoperonospora cubensis is a highly destructive downy mildew pathogen that re-emerged in 2004 and caused devastating losses to the cucurbit industry in the USA (Holmes et al. 2015) (Fig. 1). Every year since, widespread cucurbit crop failures have occurred throughout the country, which has positioned cucurbit downy mildew (CDM) as the primary threat to cucurbit production. In Europe, where CDM has challenged the cucurbit industry since 1985, annual yield losses of up to 80% are not uncommon (Cohen et al. 2015). CDM had not been a limitation for cucumber growers in the USA since the 1950s because commercial cultivars were bred to be genetically resistant to the disease (Ojiambo et al. 2015), and while other cucurbits could become infected, the disease was successfully controlled with modest fungicide applications. Nonetheless, when P. cubensis emerged in 2004, it was already resistant to two key fungicide chemistries (mefenoxam and the strobilurins) used to control other downy mildew and oomycete pathogens (Holmes et al. 2015). Currently, the disease is controlled with intensive and costly fungicide treatments, which includes applications every 5-7 days on cucumbers and every 7-10 days on other cucurbits (Savory et al. 2011).

Control of *P. cubensis* is challenging due to its ability to quickly overcome control measures such as host resistance and fungicides, and its long-distance dispersal capabilities (Ojiambo et al. 2015). The pathogen is believed to survive the winter in regions below the 30th latitude, such as southern Florida, and disperse yearly towards northern states once temperatures are warmer and susceptible hosts such as cucumber, watermelon, cantaloupe, squash, pumpkin, zucchini and gourd are available (Ojiambo et al. 2015). In addition, wild and weedy hosts have been identified in the USA (Wallace et al. 2014, 2015b) and Europe (Runge & Thines 2009), which could provide a natural reservoir for P. cubensis in addition to the 'green bridge' provided by year-round production of cucumbers in greenhouses (Holmes et al. 2015). Airborne dispersal can be problematic for disease control, as virulent or fungicide-resistant isolates can quickly spread throughout the USA. For example, data from Georgia efficacy trials during 2012 on cucumber showed reduced disease control with fluopicolide (Langston & Sanders 2013), a relatively new and key fungicide that provided excellent disease control in previous years. The following year, data from North Carolina (Adams & Ouesada-Ocampo 2014) and Michigan (Hausbeck & Linderman 2014) showed that



Fig. 1 (Colour online) Cucurbit downy mildew caused by *Pseudoperonospora cubensis*. (a) Infection on a cucumber leaf; (b) Abundant pathogen sporulation on the underside of the cucumber leaf; (c) Infection on a watermelon leaf; and (d) Little pathogen sporulation on the underside of the watermelon leaf.

fluopicolide was no longer effective for downy mildew control in cucumber.

To reduce the window of fungicide sprays and minimize disease control costs for growers, the Cucurbit Downy integrated pest management Pest Mildew (CDM) Information Platform for Extension and Education (ipmPIPE) was created (Ojiambo et al. 2011). The goal of the CDM ipmPIPE is to provide forecasting of the disease and alerts of confirmed outbreaks so that growers can initiate sprays when the disease is found in surrounding states; to date, it has saved growers 2-3 sprays per growing season (Ojiambo et al. 2011). A limitation of this system is that it relies on reports of infected plants that are diagnosed when the disease is advanced enough for visual identification. This is an important challenge since fungicides are most effective when applied preventatively at the early stages of infection when the disease is most difficult to diagnose (Savory et al. 2011). In addition, visual diagnosis requires significant training of both growers and extension personnel, and does not vield quantitative data on inoculum or its origin, which can provide valuable epidemiological insights for disease control (Quesada-Ocampo et al. 2012; Granke et al. 2013; Naegele et al. 2016; Wallace & Quesada-Ocampo 2017). While growers are very familiar with the disease in cucumber due to the characteristic angular lesions and pathogen sporulation (Fig. 1b), diagnosis of the disease in hosts such as watermelon is not straightforward since pathogen sporulation is low and lesions resemble those of other diseases (Fig. 1d) (Withers et al. 2016). Developing biosurveillance tools for early and accurate diagnostic of P. cubensis as has been done for other oomycetes (Martin et al. 2012) would significantly improve timely deployment of disease control measures. Expanding biosurveillance to be used not only for pathogen detection but also for pathogen quantification, establishing crop risk, and determining which fungicides will perform best for controlling an outbreak will allow for precise applications of chemical control as far as timing, crop target and fungicide product to use. Here, we discuss how genomics can enable development of biosurveillance tools for P. cubensis, and how such efforts need to be guided by knowledge of pathogen biology and the platform to implement detection assays so that end users can adopt them.

### Next-generation sequencing for rapid development of diagnostic tools for *P. cubensis*

Next-generation sequencing (NGS) technologies have reached a point where total DNA from diverse organisms can be analysed for unique regions or sequence variation in nuclear DNA or other highly abundant target regions such as organelle genomes (Cronn et al. 2012). Lowdepth survey of high-copy targets (Straub et al. 2011) or high-depth sequencing for unique genome fractions (Withers et al. 2016) is increasingly becoming economically feasible and technologically achievable for species identification as well as phylogeography studies (Cronn et al. 2012). From NGS data, molecular markers can be developed in almost any organism from RNA-seq and/or DNA-seq using *de novo* assembled transcriptome and/or genome, respectively (Paszkiewicz & Studholme 2010; Ekblom & Galindo 2011), thereby reducing the need for high-quality 'finished' genomic resources.

In recent years, progress has been made towards developing the baseline knowledge needed to design speciesspecific molecular diagnostic tools for P. cubensis. A draft genome assembly was generated from a cucumber isolate from Ohio (Savory et al. 2012b), host-pathogen transcriptome analyses have been completed for cucumber infections (Tian et al. 2011; Adhikari et al. 2012; Savory et al. 2012a) and population structure analyses of P. cubensis isolates from the USA and Europe were published (Quesada-Ocampo et al. 2012; Kitner et al. 2015; Summers et al. 2015b). This information has considerably increased our understanding of this pathogen's biology and host adaptability. Initial population structure analyses revealed significant diversity within P. cubensis isolates affecting cucurbit crops in the USA; isolates frequently found infecting cucumber were genetically different from those infecting other cucurbits (Quesada-Ocampo et al. 2012). These findings have been recently corroborated and expanded using hundreds of P. cubensis isolates infecting commercial and wild hosts in the USA. These results showed that some isolates are adapted to cucumber (Cucumis sativus), cantaloupe (Cucumis melo) and buffalo gourd (Cucurbita foetidissima), while others are adapted to watermelon (Citrullus lanatus), squash (Cucurbita pepo), pumpkin (Cucurbita maxima), bitter melon (Momordica charantia) and balsam apple (Momordica balsamina) (Wallace & Quesada-Ocampo 2016). The genetic structure of *P. cubensis* has important implications for diagnostics since assays need to be robust enough to detect all genetically diverse isolates of P. cubensis.

An additional challenge for *P. cubensis* molecular diagnostics is achieving specificity. Since *P. cubensis* is an obligate pathogen and thus requires a living host to survive and reproduce (Savory et al. 2011), DNA samples of the pathogen frequently also contain DNA from the host and other leaf epiphytes; thus, diagnostic assays must be highly pathogen-specific to avoid non-specific background amplification. Fortunately, the genomes of several hosts of *P. cubensis*, including cucumber (Huang et al. 2009), watermelon (Guo et al. 2013) and melon (Garcia-Mas et al. 2012) have been sequenced, which can

facilitate design of markers with improved specificity when P. cubensis is found infecting these hosts. Closely related species of P. cubensis can also complicate speciesspecific diagnosis, especially in regions where crops affected by related downy mildew pathogens are produced in contiguous geographic areas. For example, Pseudoperonospora humuli, a closely related sister species of P. cubensis, may have overlapping geographic ranges, although it causes downy mildew only on hops. In the western part of North Carolina, cucurbits and hops are grown in the same production regions. Additionally, these two sister species of Pseudoperonospora share almost identical genetic regions, such as the internal transcribed spacer (ITS) of the ribosomal gene cluster and the mitochondrial gene cytochrome C oxidase (cox) which are most commonly used for molecular diagnosis (Choi et al. 2005; Summers et al. 2015b). Another downy mildew pathogen infecting cucurbits, Plasmopara australis, has been reported to infect wild host species such as balsam apple (Echinocystis lobata) and burr cucumber (Sicvos angulatus) in some states of the USA. (Preston and Dosdall 1955). While reports of P. australis infecting commercial cucurbits are limited in the USA (Wallace et al. 2015a), the wild hosts it can infect are common weeds in the southern USA. The phylogenetic relationship of P. australis with P. cubensis and P. humuli that have either shared hosts or geographic areas, and its potential to challenge species-specific diagnostics of P. cubensis, is unclear. Clarifying phylogenetic relationships of downy mildew pathogens is the first step to developing species-specific diagnostic methods (Thines et al. 2009). The next step is to develop additional genomic resources for downy mildew pathogens that would allow understanding of the species genetic diversity and for delineating closely related species, thereby helping speciesspecific detection (Derevnina et al. 2015; Sharma et al. 2015). The current genome assembly of P. cubensis is not of finished quality and its obligate nature makes it difficult to obtain high molecular weight and pure DNA free from host and phyllosphere microbiota DNA. Available NGS technologies could greatly improve the assembled genome quality for high-throughput marker detection studies (Withers et al. 2016). Single Molecule Real Time (SMRT) sequencing by Pacific Biosciences (PacBio, Menlo Park, CA) eliminates any PCR amplification step and provides long read genomic sequences that would greatly complement and improve existing P. cubensis genome quality (Rahman, Quesada-Ocampo, unpublished).

By combining computational approaches with NGS, highly specific molecular diagnostic markers (even host lineage specific) can be effectively designed based on single nucleotide polymorphisms (SNPs) or allelic polymorphism or presence/absence of exons/epitopes of specific genes/proteins (Summers et al. 2015b; Withers et al. 2016). Recently, through use of NGS and bioinformatics tools, multiple species-specific candidate genomic markers were identified in P. cubensis (Withers et al. 2016). The study entailed sequencing of multiple P. cubensis and P. humuli isolates from diverse hosts, followed by comparison of the RNA-seq data to identify unique regions conserved in P. cubensis. The distinction between these two species has been subject to much debate due to their morphological similarities as well as high sequence identity (Choi et al. 2005; Summers et al. 2015b). Therefore, comparison among multiple isolates from both species was required to carefully select unique genetic regions. shared exclusively by all isolates of P. cubensis but absent in P. humuli isolates, that could be developed as diagnostic markers (Fig. 2). These candidate markers were also evaluated against multiple isolates of P. cubensis from all over the world as well as other oomycetes to confirm their specificity and allow their use in molecular diagnostics through real-time PCR techniques (Rahman & Quesada-Ocampo 2016). One additional advantage of developing markers from transcriptome sequences is that such markers are associated with functional genes and could be used for pathogen detection in combination with studies of host-resistance breakdown and/or host-adaptation (Wallace & Ouesada-Ocampo 2017). Future biosurveillance of high-risk plant pathogens, like P. cubensis, will be enhanced by the development of portable DNA sequencing devices to determine the aerial load as well as genetic diversity of airborne pathogens. Integration of such high-throughput DNA sequencing technologies with portable microfluidic and lab-on-chip platforms, in the form of biosensors, to detect multiple plant pathogens for on-site use by unskilled users, will be the key for robust plant pathogen diagnostics and biosurveillance (Nezhad 2014; Ray et al. 2017).

### Developing early detection tools for cucurbit downy mildew

The obligate biotroph *P. cubensis* disperses large numbers of airborne spores from one susceptible host to another for both survival and spread since the pathogen is completely dependent on living host tissue for reproduction (Naegele et al. 2016). Additionally, extinction-recolonization cycles (Brown & Hovmoller 2002), dormant stage through sexual reproduction or re-establishment from external sources, have been recorded in *P. cubensis* isolates from China, Japan, India, Europe and the Middle East (Bains & Jhooty 1976; Lebeda & Cohen 2011;



Fig. 2 (Colour online) Identification of species-specific nuclear regions in *Pseudoperonospora cubensis* through comparative genomics with sister species *P. humuli* using next-generation sequencing. 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.

Cohen & Rubin 2012; Zhang et al. 2012), as well as in other downy mildew pathogens such as tobacco blue mould (*Peronospora tabacina*) (Aylor 1999; Mahaffee 2014). Following the major outbreak of CDM in the USA in 2004, several research initiatives have been undertaken to investigate the epidemiology and aerobiology of pathogen dispersal from overwintering sources. Early warning through detection of *P. cubensis* airborne sporangia presence has also been recognized as a major grower and stakeholder priority (Holmes et al. 2015).

Since 2006, a series of Burkhard volumetric spore traps have been established in Michigan to serve as a tool to alert growers to the presence of airborne sporangia of P. cubensis (Granke & Hausbeck 2011). Spores are impacted on adhesive tape on hourly, daily or weekly time intervals that can later be stained and counted under a microscope. A defined threshold of spores per week was used, along with information from the CDM ipmPIPE, to determine when it was prudent to begin fungicide sprays (Granke et al. 2013). A spore-trapping study detected P. cubensis sporangia within a few days of trapping initiation in early June in Michigan during 2006, 2007 and 2009, indicating that inoculum was probably present before sampling began (Granke & Hausbeck 2011). Another study found that airborne sporangia concentrations were significantly associated with downy mildew occurrence (Granke et al. 2013), confirming that spore traps could be used as an early detection system for cucurbit downy mildew. However, a significant drawback of the traditional tape spore traps that has limited their deployment is the time and labour that must be invested in adding the adhesive to the tape for sampling, cutting the tape into slide-size pieces for counting, staining the pieces of cut tape to identify viable sporangia, and counting the sporangia under a microscope. Also, since this process is based on visual identification, there is a significant risk of misidentifying sporangia from other downy mildews as that of P. cubensis, especially if several downy mildews occur in the same region and growing season (Klosterman et al. 2014). However, capturing aerial sporangia of *P. cubensis* using rotorod type impaction spore traps has also been documented (Summers et al. 2015a), which paved the way to utilize such spore traps in conjunction with quantitative real-time PCR like that of other downy mildew pathogen detection systems, e.g. Peronospora effusa (Klosterman et al. 2014) and Bremia lactuace (Kunjeti et al. 2016).

Combining quantitative molecular diagnostics with spore traps could provide specific, effective and sustainable early detection for airborne oomycete pathogens that is not dependent on disease outbreak reports. Spore traps that collect air samples at different time intervals which are then stored in microcentrifuge tubes are commercially available (e.g. the multi-vial cyclone spore sampler of Burkard Manufacturing Co., Ltd, Hertfordshire, UK). Since the system would rely on sporangia levels to issue an alert to spray fungicides, the sporangia would need to be quantified by molecular methods, with the underlying assumption that DNA concentration would be correlated with sporangial concentration. A challenge with using

DNA as a proxy for sporangia detection in contrast to direct staining and counting viable sporangia that absorb the dye, is that the viability of the sporangia is unknown. Also, the DNA of sporangia in these samples would need to be extracted or made available for PCR amplification in a consistent way across samples that removes PCR inhibitors or in a way that no DNA is lost, e.g. by using enzymatic extraction protocols (Fredricks et al. 2005). Lastly, most molecular diagnostic tools for oomycetes have been based on markers such as the ITS and mitochondrial genes, which may have copy number variation from isolate to isolate and thereby possibly confound sporangia quantification (Klosterman et al. 2014; Kunjeti et al. 2016). Preference for these markers is in part due to the lack of genomic resources for airborne oomycetes that would allow design of species-specific markers. While thousands of nucleotide sequences are available in GenBank for airborne oomycetes, most of these are related to ITS or mitochondrial genes and represent well-studied species such as Phytophthora infestans. Generating genomic resources for understudied airborne oomycetes, such as the downy mildews and some Phytophthora species, will be needed to develop markers better suited for sporangia quantification via spore traps (Withers et al. 2016).

Previous studies used a combination of spore traps and quantitative PCR for species-specific detection of the downy mildew pathogens of spinach (P. effusa) and beet (Peronospora schachtii) (Klosterman et al. 2014), lettuce (Bremia lactucae) (Kunjeti et al. 2016), cucumber (P. cubensis) (Summers et al. 2015a) and hops (P. humuli) (Gent et al. 2009; Summers et al. 2015a). These studies relied on ITS-based or mitochondrial markers for detection and quantification of the downy mildew pathogens and sometimes encountered non-specific amplification of closely related species. Nonetheless, most of these studies successfully quantified sporangia of the target pathogens with high sensitivity and the sporangia quantification using real-time PCR was positively correlated to visual spore counts (Gent et al. 2009; Klosterman et al. 2014). A recent study targeting a mitochondrial locus for Bremia lactucae can detect a single sporangium and was useful for quantifying the pathogen from rotorod air samplers (Kunjeti et al. 2016). On the other hand, mitochondrial marker cox2 based detection of P. cubensis and P. humuli aerial sporangia from similar rotorod samplers was reported to be inconclusive due to variable Cq values against sporangial counts (Summers et al. 2015a). Understanding the dynamics of airborne inoculum is critical, as the arrival of sporangia is the first step toward infection and epidemic initiation (Cohen & Eyal 1977). These PCR-based spore traps will be helpful in quantifying the influx of sporangia during the growing season to improve forecasting models and rapidly provide sporangia levels for timing of fungicide applications (Gent et al. 2009). Recently, progress has been made in detection of P. cubensis sporangia with high sensitivity and specificity using unique nuclear molecular markers generated using high-throughput DNA and RNA sequencing (Rahman & Quesada-Ocampo 2016; Withers et al. 2016). From these initially identified unique markers, several were later developed for detection of P. cubensis sporangia using real-time quantitative PCR. In laboratory experiments using sampling rods from rotorod type impaction spore traps (Fig. 3) inoculated with varying concentrations of P. cubensis sporangia, a detection limit of 10 sporangia was reported when using TagMan probe with LNA (locked nucleic acid) bases (Rahman & Quesada-Ocampo 2016). However, further experiments are warranted, including detection of P. cubensis sporangia captured in the field by spore traps to confirm usability of these markers for biosurveillance of aerial load of this downy mildew pathogen.

Since the innovation of the first Burkhard volumetric spore traps, numerous additional improvements and innovations have been made to the air sampling devices that utilize different mechanisms of entrapment, such as impaction (Rotorod spore samplers, ChemVol High Volume Cascade Impactor), filtration (Button, IOM), virtual impaction (Burkard Jet spore sampler, Miniature Virtual Impactor, Biral Aspect) and electrostatic attraction (Ionic spore trap) (West & Kimber 2015). For biosurveillance purposes, however, high volume spore traps, including rotorod, jet and ionic spore traps are likely to be more useful (Heard & West 2014). Developments of these air samplers were mostly made with considerations to add precision to identify pathogens using molecular techniques (West & Kimber 2015). Furthermore, with the advances in computational power and miniaturization of electronics, new technologies like autonomous mobile platforms, such as unmanned aerial vehicles (UAVs) are gaining traction in aerobiology and airborne plant disease dynamics (Gonzalez et al. 2011). For example, presence of sporangia of the potato late blight pathogen, Phytophthora infestans, was detected in the lower atmosphere (25-45 m) above infected potato canopies using autonomous UAVs (Techy et al. 2010). Application of such technology to capture viable aerial sporangia is possibly much greater for P. cubensis due to its melanized spores which can provide higher tolerance to solar radiation (Holmes et al. 2015). By integrating exponential decay function (viability to solar radiation), concentration of viable P. cubensis sporangia captured by UAV mounted spore traps could be used as a more accurate



Fig. 3 (Colour online) Rotorod impact type spore traps. (a) Rotorod air sampler unit with arms attached. (b) Grower-friendly roto-rod trap developed for monitoring *P. cubensis* airborne inoculum.

prediction of the risk of disease outbreak (Holmes et al. 2015). Real potential of such mobile sampling systems, however, depends on the integration of sophisticated technologies that can automate the capture, rapid *in situ* diagnosis and geo-reference aerial collections (West and Kimber 2015). Since CDM mostly spreads from southern states northwards (Ojiambo et al. 2015), early aerial detection and accurate identification (host specificity and fungicide resistance) of *P. cubensis* using a combination of UAV and portable identification devices using NGS technologies would revolutionize epidemiological studies as well as improve biosurveillance of this airborne pathogen.

### High-throughput monitoring of fungicide resistance in *P. cubensis*

A variety of fungicides are currently used to control *P. cubensis* after the re-emergence of the pathogen in 2004. At that time, *P. cubensis* was already resistant to some common chemistries, such as mefenoxam (Fungicide Resistance Action Committee, FRAC code 4) and quinone outside inhibitors (FRAC code 11) (Table 1) (Reuveni et al. 1980; Ishii et al. 2001; Ojiambo et al. 2015; FRAC 2017). The fungicides used to control *P. cubensis* are known to vary significantly in their efficacy, and are also affected by location, host and other environmental conditions in that particular growing season (Adams & Quesada-Ocampo 2016).

Since *P. cubensis* is an obligate foliar biotroph, evaluating fungicide resistance is extremely difficult and laborious under laboratory conditions as compared with other oomycete pathogens. Currently, evaluations of fungicide resistance are done in the field, where control efficacy is observed by looking at overall disease severity compared with previous years and other fungicide management programmes with different modes of action. To acquire accurate measurements of an isolate's EC50 value, *P. cubensis* isolates are typically cultured in a laboratory setting on leaves of the most susceptible host cucumber that are treated with various concentrations of the fungicide. Whether fungicide resistance assays are performed in the laboratory or in the field, usually observations in cucumber are extrapolated to other cucurbit hosts due to the difficulty in conducting the assays (Ishii et al. 2001; Zhu et al. 2008).

If researchers could monitor the presence of the P. cubensis pathogen and fungicide resistance alleles in the field using molecular techniques, it would be possible to optimize fungicide choice based on the resistance level that is present in that specific field. Furthermore, this strategy of active resistance monitoring could aid in a fungicide resistance management programme. Currently, some resistance mutations have been reported for both the cellulose synthase gene and the cytochrome b gene for FRAC (Fungicide Resistance Action Committee) codes 40 and 11 fungicides (Fig. 4), respectively (Ishii et al. 2001; Blum et al. 2011). Preliminary research on both mutations has shown that P. cubensis isolates that are recovered from cucumber contain these mutations at a significantly higher rate than that of isolates from other cucurbit hosts like gourds (Fig. 5). Presumably, this is due to the increased fungicide usage that is required to manage cucurbit downy mildew in cucumber fields (D'Arcangelo, Miles & Quesada-Ocampo, unpublished).

The first step in developing molecular markers to detect fungicide resistance is identifying the gene target of the fungicide. Unfortunately, many of the active

Active ingredient	Product and Manufacturer (City, State, Country)	FRAC <sup>1</sup>	Target site <sup>1</sup>	Resistance in Oomycetes?	Molecular mechanism of resistance identified in oomycetes?
Famoxadone + cymoxanil	Tanos, Dupont (Wilmington, DE, USA)	11 + 27	cyt b gene (Qo site) + unknown	Yes (Ishii et al. 2001)	G143A amino acid change in cyt B (famoxadone)
Cyazofamid	Ranman, Summit Agro USA (Cary, NC, USA)	21	cyt b gene (Qi site)	Not officially reported	Unknown
Cymoxanil	Curzate, Dupont (Wilmington, DE, USA)	27	Unknown	Not officially reported	Unknown
Propamocarb	Previcur Flex, Bayer Crop Science (Leverkusen, Germany)	28	Cell membrane permeability	Not officially reported	Unknown
Mefenoxam	Ridomil, Syngenta (Basel, Switzerland)	4	RNA polymerase I	Yes (Reuveni et al. 1980)	See note <sup>2</sup>
Mandipropamid	Revus, Syngenta (Basel, Switzerland)	40	Cellulose synthase	Yes (Blum et al. 2011, Blum et al. 2012, Zhu et al. 2008)	G1105W/V amino acid change in cellulose synthase gene (cesA3)
Fluopicolide	Presidio, Valent U.S.A. (Walnut Creek, CA, USA)	43	delocalization of spectrin-like proteins	Not officially reported	Unknown
Ametoctradin + dimethomorph	Zampro, BASF (Ludwigshafen, Germany)	45 + 40	cyt b gene (Qo site, stigmatellin binding sub-site) <sup>3</sup> + Cellulose synthase	Yes for dimethomorph component (Blum et al. 2011, Blum et al. 2012, Zhu et al. 2008)	Unknown + G1105W/V amino acid change in cellulose synthase gene (cesA3) (dimetomorph)
Mancozeb + zoxamide	Gavel, Gowan Company (Yuma, AZ, USA)	M + 22	Multi-site + ß-tubulin assembly in mitosis	Not officially reported	Unknown
Oxathiapiprolin	Orondis, Syngenta (Basel, Switzerland)	49	OSBPI oxysterol binding protein	Yes for <i>Phytophthora</i> <i>capsici</i> (Miao et al. 2016)	Amino acid change G769W PcORP1 gene found in <i>P.</i> <i>capsici</i>

Table 1. Fungicides used to control *P. cubensis* sorted by active ingredient, commercial trade name, FRAC group, mode of action in the fungicide resistance action committee, and whether molecular markers are currently available to detect this resistance.

<sup>1</sup>For more information, check the FRAC mode of action list (FRAC 2017).

<sup>2</sup>Resistance mechanism has been reported in *Phytophthora infestans*. Single nucleotide polymorphisms have been identified in the RNA polymerase I gene (Randall et al. 2014; Matson et al. 2015).

<sup>3</sup>Cross resistance not typically reported for fungicides that also affect cyt b such as FRAC codes 11 and 21, probably a different mechanism of resistance.

ingredients that are used to control P. cubensis (e.g. cymoxanil, flucopicolide, propamocarb) do not have specific targets, even though resistance is suspected in many cases (FRAC 2017). To identify these regions, clear phenotypes need to be established between a sensitive and a resistant isolate through laboratory evaluations using various concentrations of the fungicide. Afterwards, a variety of gene identification and sequencing technologies can be employed to identify potential SNPs that are associated with resistance. In a study on *Phytophthora infestans*, genetic crosses and cloning of candidate genes were used to identify a specific locus, RPA190, which has 86% association with mefenoxam insensitivity (Randall et al. 2014). Research is required to determine whether this candidate and the associated SNPs could be transferred to P. cubensis. For less characterized fungicides, a comparative genomics pipeline on both DNA and RNA data on isolates with different levels of sensitivity should prove effective in identifying candidate SNPs, especially when there is a known gene target or mechanism.

Once a locus has been identified, there are several novel detection techniques that can be employed to detect fungicide resistance alleles. The cornerstone technique is performing PCR on single sporangial isolates of P. cubensis followed by Sanger-based sequencing, to establish the fungicide resistance diagnostics assay. To confirm specificity, a range of isolates of P. cubensis should be tested along with closely related species (e.g. P. humuli or Phytophthora capcisi). Once defined, the assay can easily be adapted to other PCR techniques commonly utilized to detect SNPs. Some examples of these techniques include amplification refractory mutation system-PCR (Baudoin et al. 2008), allelic discrimination using TaqMan PCR, and SYBR Green based-high resolution melt curve analysis (Summers et al. 2015b). These techniques have the capability to discriminate SNPs, and probe-based TaqMan techniques are probably the most straightforward but do require optimization. One challenge is that assays need to be run at relatively higher temperatures, which reduces overall sensitivity.



**Fig. 4** (Colour online) Visualization of well-characterized single amino acid changes that result in fungicide resistance in *P. cubensis*. (a) Cellulose synthase 3 gene (CesA3) with an alteration from a glycine to a tryptophan or valine at the 1105th amino acid for FRAC code 40 fungicides (e.g. dimethomorph). (b) Cytochrome b gene (cyt b) with an alteration from a glycine to an alanine at the 143rd amino acid for FRAC code 11 fungicides (e.g. quinone outside inhibitors).



Fig. 5 Fungicide resistance in *P. cubensis* found on various cucurbit hosts collected throughout North Carolina from 2012–2014. Solid bars denote resistant isolates that contain fungicide mutations associated with resistance, where clear bars denote sensitive isolates without the mutations. (a) Isolates that contained CesA3 gene mutations (G1105V/W). (b) Isolates that contained cyt B gene mutations (G143A).

A shortcoming of PCR-based strategies is that in field samples, multiple alleles and isolates can be present in a single environmental sample, some with potentially different fungicide resistant alleles. Usually the limit of detection of a fungicide resistant allele in a mixed sample is about 5–10% (when mixed with DNA containing sensitive alleles), when using probe-based TaqMan strategies to discriminate SNPs (T. Miles, unpublished). Other techniques are currently available which allow for finer resolution in these environmentally mixed samples, such as AmpSeq and Digital Droplet PCR. AmpSeq is a NGS technology that is currently used to rapidly genotype many individuals; the process to collect and analyse the data is not rapid but does allow for extremely detailed counts of alleles of interest (Yang et al. 2016). Another approach is a new form of PCR known as Digital Droplet PCR. This system separates a single PCR reaction into up to 20 000 droplets, each with their own amplification that is monitored at the end by a microfluidic droplet reader to determine which droplets had amplification by measuring fluorescence (Fig. 6). This technology is not inexpensive to set up but has many potential applications in detecting resistant alleles in an extremely mixed sample, which may be common on a spore trap sampling rod. Recently, this technology is being used to study genotypes of the impatiens downy mildew pathogen *Plasmopara obducens* in natural populations of the pathogen (J. Crouch, unpublished).



2-dimensional digital droplet PCR for detecting the G143A mutation in the cytochrome b gene

Probe 2 for A143 allele

Fig. 6 (Colour online) Digital Droplet PCR to detect an amino acid change in the cytochrome b gene in *P. cubensis* associated with fungicide resistance to quinone outside inhibitor fungicides (FRAC code 11). Note that in this figure two *P. cubensis* isolates are shown together, one with the sensitive mutation (G143) and one with the resistant mutation (A143) to demonstrate various clusters of droplets associated with either allele.

Another potential method is the development of isothermal diagnostics to detect single nucleotide polymorphisms in a rapid, inexpensive manner. Currently, the most common isothermal techniques are Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) (Craw & Balachandran 2012). LAMP is by far the most widely published isothermal detection for oomycetes and assays have been developed for *Phytophthora* and Pythium spp. (Tomlinson et al. 2010; Fukuta et al. 2014). RPA has also been used in a systematic fashion to detect various Phytophthora and Pythium spp. using an interchangeable probe-based marker system (Miles et al. 2015, 2016). When using these techniques to detect fungicide resistance, there are several challenges that need to be addressed. For example, the polymerase used in LAMP lacks exonuclease activity, making it less effective with traditional TaqMan type probes. As a result, new FRETbased assimilating probes are required which differ in their design to detect amplicons generated in a LAMP reaction. However, effectiveness of such probes designed to discriminate SNPs needs to be investigated (Kubota & Jenkins 2015). Similarly, primer and probe specificity for RPA assays have been questioned when amplifying sequences with only minor differences (Daher et al. 2014). A solution to these problems could include altering primer/probe concentrations, or running competitive RPA assays (T. Miles, unpublished).

Future technologies that can accurately discriminate SNPs in a rapid manner and are extremely quantitative will be able to overcome some of the current limitations with PCR-based technologies to allow researchers to more accurately detect SNPs in environmental samples. The real challenge to overcome is the identification of gene targets and SNPs associated with resistance of newer classes of fungicides used to control *P. cubensis* to develop new markers to detect resistance.

## Mitochondrial genomes: a source of conserved, species-specific or lineage-specific markers for biosurveillance of *P. cubensis*

Mitochondrial genomes have several advantages for development of diagnostic and population markers. These rapidly evolving genomes exhibit sequence polymorphisms capable of differentiating even closely related species, and their high copy number relative to the nuclear genome also improves sensitivity of the assays. Due to their relatively small size in oomycetes (generally under 70 kb), they are also easy to assemble using Illumina data (but due to the comparatively high level of homopolymeric repeats that are present in mitochondrial genomes, assemblies from 454 data are prone to errors). To develop a systematic approach for diagnostic marker development for oomycetes, a project was initiated to assemble the mitochondrial genomes for a range of taxa (F. Martin, unpublished). To date, over 450 genomes have been assembled representing 13 genera and 87 species, including a range of downy mildew pathogens, Phytophthora, Pythium, Aphanomyces and several other genera. The gene content among taxa is similar, with large and small ribosomal RNAs, 35 mitochondrial genes and a suite of tRNAs. In addition to these conserved genes, there are also several putative open reading frames (ORFs), some of which are conserved among all taxa while others are unique for a species.

This large dataset provides an opportunity to use comparative genomics to identify target loci and perform in silico analysis as a preliminary screen for specificity. One approach for marker design that has been particularly helpful for enhanced specificity is to identify a unique gene order present in a genus, thereby reducing the need for high stringency during amplification to maintain specificity. For example, a multiplexed genus and speciesspecific assay for Phytophthora targeted primer annealing sites that spanned a unique gene order that were less than 450 bp apart, whereas they were 15 kb apart in the related genus Pythium and much further apart in plants and Eumycotan fungi (Martin & Quesada-Ocampo, unpublished). The resulting assay had two diagnostic markers. one that was only 'genus' specific, and the other that was both 'genus' and 'species' specific within a single amplicon (Bilodeau et al. 2014). Subsequent work has shown the same gene order in many downy mildew species, but for most taxa sequence polymorphisms are present in analogous primer annealing sites (F. Martin, unpublished). More recent work has increased the total number of species-specific TagMan probes validated from 14 (Bilodeau et al. 2014) to 46 taxa and in silico analysis suggests species-specific probes could be developed for 89% of the 145 taxa investigated (Miles et al. 2017). Targeting gene order differences has also been successful for designing a genus-specific marker system for Pythium (Miles & Martin, unpublished) and in silico analysis indicates this is possible for Aphanomyces as well (F. Martin, unpublished). Another approach for marker design is to target a unique putative ORF; this was used to design a species-specific mitochondrial marker for Bremia lactucae (Kunjeti et al. 2016). The marker was highly sensitive, capable of detecting a single sporangium with a  $C_{\alpha}$  of 32, which is below the cut-off for linearity of amplification. A similar approach for marker design was used for development of an assay for Peronospora effusa (Kunjeti, Martin & Klosterman, unpublished).

Some downy mildew pathogens, including *P. cubensis*, have a similar gene order as *Phytophthora* but sequence polymorphisms have been identified that will allow for differentiation of host-specific isolates of *P. cubensis* from other taxa, including the closely related *P. humuli* (F. Martin, unpublished). Two different markers were designed that can separate host-adapted isolates of *P. cubensis* (cucumber, cantaloupe and buffalo gourd compared with watermelon, squash, pumpkin, bitter melon and balsam apple) using conserved primer annealing sites flanking a large indel, such that amplicon size differences could differentiate crop risk groupings (Rahman, Martin & Quesada-Ocampo, unpublished). A TaqMan diagnostic assay is currently under development targeting a region with a smaller indel that should enable a multiplexed detection of host-adapted isolate groupings of *P. cubensis* as well as *P. humuli* (F. Martin, unpublished).

Aside from providing a resource for developing diagnostic markers, having a database of assembled mitochondrial genomes provides a resource for development of additional markers useful in population studies. While amplification and sequencing of specific loci can be useful for identification of polymorphisms and classification of mitochondrial haplotypes, the ability to conduct whole genome comparisons provides a more comprehensive evaluation of loci. For example, comparison of 10 mitochondrial genomes of P. cubensis identified four mitochondrial haplotypes and facilitated design of primers that should be useful for amplification of specific loci for sequence analysis from additional isolates for a broader assessment of haplotype diversity (F. Martin, unpublished). This approach also has been useful for mitochondrial haplotype analysis in Phytophthora (Martin & Coffey 2012; Mammella et al. 2013).

Comparative mitochondrial genomics is also useful for in silico evaluation of specific genes for taxonomic and phylogenetic purposes (Martin et al. 2014). While working with obligate pathogens, the genome alignments could also provide guidance to design highly conserved and specific primers to reduce problems with amplification of non-target organisms in environmental samples. These alignments would be particularly helpful when designing markers for metagenomic studies across broader taxonomic classifications. For example, the primers used for amplification of the *rps10* locus in a phylogenetic study of Phytophthora (Martin et al. 2014) were designed from highly conserved regions of tRNAs flanking the rps10 gene and should be useful for amplification of of the Peronosporales, **Pvthiales** members and Saprolegniales (F. Martin, unpublished). Owing to their smaller size and multicopy presence as well as being clonal, neutral and with a clock-like evolutionary rate, mitochondrial markers have been reported to be ideal for population studies and biosurveillance for pathogen diversity (Galtier et al. 2009).

### Current and future applications of DNA-based technologies for biosurveillance

With the reduction of extension personnel in many US states, commercial growers are starting to rely more on centralized plant clinics and agricultural consulting companies for diagnostics of diseased samples (Miller et al. 2009). User-friendly diagnostics for people with minimal training is becoming more of a necessity in today's agriculture. Downy mildew pathogens are mostly diagnosed by visual inspection of infected tissue and identification of pathogen structures, such as sporangiophores bearing sporangia. However, pathogen sporulation, which is the key identification characteristic for downy mildew pathogens, is not always obvious on every host and downy mildew symptoms can frequently resemble other foliar diseases. Furthermore, in some hosts, downy mildew pathogens can be seedborne (basil downy mildew) or systemic (hop downy mildew) and produce no visible symptoms until conditions are favourable (Gent et al. 2009). Therefore, molecular diagnostic methods for biosurveillance of downy mildew pathogens need to be extremely robust against an overwhelming background of non-target DNA. Grower-friendly diagnostics such as immunostrip of Agdia for Phytophthora, lateral flow device 'Alert kit' from Neogen Corp for Pythium have been available; however, no such resources exist for downy mildew pathogens. Although a lateral flow device (LFD) using a monoclonal antibody against the onion downy mildew pathogen Peronospora destructor was developed (Kennedy & Wakeham 2008), its detection sensitivity was much less (500 sporangia) than needed ( $\leq 10$  sporangia) to be an effective and practical biosurveillance system (Mahaffee 2014). Recent progress in utilization of NGS data to identify unique biomolecular markers of P. cubensis in conjunction with a real-time PCR detection system holds immense potential for the development of highly specific and sensitive detection systems (Rahman and Quesada-Ocampo 2016; Withers et al. 2016). Furthermore, since the unique markers were identified using RNA-seq data, the potential of such markers to be used in developing immunosensor strips or other types of LFD or biosensors in the future is enormous.

Laboratory methodologies for detection and quantification of airborne inoculum are improving at a fast pace. The real challenge, however, lies in the transfer of sophisticated laboratory techniques to field applications that can be used by unskilled workers. A successful example is Cepheid SmartCycler, which transferred methods for real-time PCR-based detection of Phytophthora ramorum from conventional research laboratory assays to field detection (Ray et al. 2017). Rapid progress of NGS technologies is certainly promising for future development of more efficient portable devices for field application. PCR-based enrichment, together with high-throughput NGS platforms integrated with microfluidic systems, could be one of the key features of future portable devices for on-site pathogen detection (Nezhad 2014). A major impediment of delivering such devices is sample preparation and DNA extraction from the pathogen as well as eliminating any PCR/DNA sequencing inhibitors. Technologies including a nanorod based cell lysis, dielectrophoresis (DEP), micro/nano particle and membrane-based DNA and RNA separation, and amplification (e.g. loopmediated isothermal amplification (LAMP)) on microfluidic and lab-on-chip devices provide promising progress in this field (Cheong et al. 2008; Kim et al. 2010; Liu et al. 2011; Sonnenberg et al. 2013). Isothermal amplification diagnostic methods, such as RPA combined with LFD, which has gained significant progress in plant pathogen detection (Zhang et al. 2014), would be especially useful in downy mildew diagnostics due to their high level of specificity (DNA-based) and field-friendly nature (De Boer and Lopez 2012). On the other hand, biosensor based detection systems, e.g. surface plasmon resonance (SRP) immunosensors based detection of Phytophthora infestans and SPR immunosensor based on DNA hybridization for detection of fungal pathogens (Fusarium culmorum) with high sensitivity, have been reported (Skottrup et al. 2007; Ray et al. 2017). Combining mobile platforms, such as UAVs accommodating a miniaturized high-throughput DNAbased detection system and/or novel biosensors for detection of airborne downy mildew sporangia that is integrated in real time with modelling systems for pathogen dispersion, could unlock the full potential of NGS technologies for P. cubensis biosurveillance in the future. Reliable and integrated biosurveillance methods that provide information about presence or absence of the pathogen, amount of inoculum, crop risk, time to initiate fungicide applications, and effective fungicides to use will guide precision cultural and fungicide management practices for control of cucurbit downy mildew (Mahaffee 2014; West & Kimber 2015).

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