Understanding Emerging Races of Basil Downy Mildew (*Peronospora belbahrii*) Using Highly Conserved Barcoding Regions

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Abstract

Basil downy mildew (*Peronospora belbahrii*) is a biotrophic oomycete pathogen which infects basil plants globally. Since its arrival in the United States in 2007, basil downy mildew has devastated both commercial and home-grown basil crops, due to its ability to spread rapidly, and persist in greenhouse environments. In recent years, we have observed *P. belbahrii* isolates which can infect different basil resistant cultivars, indicating a higher degree of genetic diversity in the population than previously predicted. To provide an overview of population genetic drive, the internal transcribed spacer (ITS) rDNA regions of three isolates of interest were amplified, sequenced, and compared to a previously assembled data set. These data were combined with a cultivar panel, where pathogenicity of each isolate was compared across ten basil cultivars. Our phenotypic observations were reflected in the phylogenetic relationships between the three pathogen isolates, indicating a connection between pathogen race and ability to overcome cultivar resistance mechanisms. Understanding how these genomic changes are reflected in pathogenicity will allow future research groups to more effectively design resistant basil cultivars and provide a quick way to identify which *P. belbahrii* strain is present in a specific crop.

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List of Abbreviations

ITS: Internal transcribed spacer

rDNA: Ribosomal DNA

PAMP: Pathogen associated molecular patterns

PTI: PAMP-triggered immunity

ETI: Effector-triggered immunity

PCR: Polymerase chain reaction

rRNA: Ribosomal RNA

DI: Disease incidence

gDNA: Genomic DNA

Introduction

Sweet basil (*Ocimum basilicum)*, a cultural and culinary cornerstone throughout the United States and the world, is best known for its unique smell and taste. Cultivated globally by home and industry growers alike, its economic and agricultural importance are clear. Less clear, however, are management strategies for basil downy mildew (*Peronospora belbahrii*), which causes significant basil crop losses every year.

Peronospora belbahrii is an oomycete biotrophic obligate plant pathogen. This means that it requires a compatible, living plant host to survive and reproduce. Downy mildew pathogens in the *Peronospora* genus are often defined by the plants which they can successfully infect (Rivera et al., 2016). Additionally, researchers have compared highly conserved genome regions known as inter-transcribed spacers (ITS), in the rDNA to differentiate *Phytophthora* species, an oomycete genus similar to *Peronospora* (Crawford et al., 1996). This technique was later used in 2005 to formally recognize the emergence of a previously undescribed basil downy mildew pathogen, *Peronospora belbahrii* (Belbahri et al., 2005). Basil plants infected by this oomycete display dark grey-brown, furry growth on the underside of leaf tissue, which is the oomycete itself, as well as leaf wilting or tissue necrosis. This specific pathogen grows best in environments with high humidity and cool temperatures (Belbahri et al., 2005). In the years since its formal classification, *Peronospora belbahrii* has continued to infect the basil populations of home growers and professional farmers and growers.

Basil downy mildew, like many plant pathogens, utilizes small, secreted proteins, described as effectors, to manipulate the immune systems of their hosts. These effector proteins

can be targeted to one of two plant immune responses. Pathogen associated molecular patterns (PAMP) are specific molecules recognized by receptors on the hosts cells surface, causing PAMP-triggered immunity (PTI) (Dodds and Rathjen, 2010). Alternatively, the presence of effector proteins in a host plant may cause an effector-triggered immunity (ETI) response. Often the receptors which cause the triggering of ETI are highly-specific to the effector proteins used by different pathogens (Dodds and Rathjen, 2010). While plant hosts continue to adapt better effector recognition receptors, pathogens continue to adapt effector proteins which can evade detection, and actively suppress the immune response of the host.

Secreted effector proteins play an important role in the infection process of many plant pathogens. These effectors are often secreted into the space between the plant cell membrane and cell wall (apoplast), or into the cytoplasm itself, where they play a role in inhibiting the hosts immune response (Dodds and Rathjen, 2010). Recently, researchers have begun to investigate the specific role of some effectors in the host colonization process (Lopez et al., 2019). These analyses investigate the localization of the effector in the host plant, and sometimes can determine their function from homology of known proteins and studying the mechanisms of host immunity.

Officially described in 2005, *Peronospora belbahrii* makes use of several effector proteins to facilitate the colonization of basil plants (Belbahri et al., 2005). Although this *Peronospora* species was originally described, in part, due to its colonization of the host plant *Ocimum basilicum,* recent years have seen a diversification in hosts for this plant-pathogen (Rivera et al., 2016). This host diversification, as well as reports of new resistance-evading *Peronospora belbahrii* races suggests significant genetic divergence within the pathogen population (Ben-Naim and Weitman, 2021). Previously, our project investigated eleven specific

effector proteins across our *P. belbahrii* population, as identified by metatranscriptomic survey (Guo et al., 2016). Using PCR, the genes coding for these proteins were isolated and sequenced. Most recently, we have identified three phenotypically distinct isolates, which each successfully colonize a different combination of resistant basil cultivars. Of these eleven effector proteins, each amplify similarly and have high sequence similarity in each of the three *P. belbahrii* isolates. This suggests that the mechanism for this phenotypic difference lies outside the sequences for these specific proteins.

To further investigate the source of these different phenotypes, we sequenced and compared the ITS regions of each of the three isolates. Previous literature indicates that as compared to other plant pathogens, *Peronospora belbahrii* has a small secretome, requiring us to look beyond our effectors of interest when comparing population members (Thines et al., 2020). The first step of looking beyond these specific proteins is understanding the overall genetic diversity of our samples. For many eukaryotes, comparison of the 18s rDNA is sufficient to determine speciation (Smit et al., 1999). This gene codes for the small ribosomal subunit in eukaryotes. It is highly enough conserved that even small changes in this region can indicate a high level of genetic drift. However, this level of detail is still too imprecise to understand the proposed races of *P. belbahrii*.

Even more highly conserved than the 18s rRNA gene are the Internal Transcribed Spacers, also located in the rDNA. These ITS regions are often used to differentiate different races of the same oomycete or fungal species (Voglmayr, 2003). The conservation of these regions across isolates allows for small sequence differences to be extrapolated into diverging races. Connecting how these pathogen genomes are changing with their host colonization phenotypes is important for understanding how the global population of *P. belbahrii* is adapting

to the presence of resistant cultivars. In this experiment, we compare three phenotypically distinct isolates collected from the United States to previous ITS data from *Thines et al., 2020*.

As the most cultivated herb globally, sweet basil (*Ocimum basilicum*), is essential for the success of many commercial agricultural operations, and a cornerstone of home cooks and gardeners. Additionally, oil made from sweet basil has been shown to reduce the symptoms of colds, spams, and insect and snake bites, as well as demonstrating anticancer and antimicrobial properties (Ahmad Ch et al., 2015). However, the cultivation of this important plant is being threatened by the oomycete pathogen *Peronospora belbahrii.* Control methods for this downy mildew are often expensive and highly toxic (Gisi, 2002). Even when chemical controls do prevent downy mildew outbreaks, they significantly contribute to the development of resistant subpopulations (Gisi, 2002). These control methods likely play a role in the phenotypic differences in host colonization that growers have begun to observe.

Recently, more work has been done to identify the resistance genes in basil that play a role in preventing successful colonization by *P. belbahrii,* as well as the development of new resistant cultivars (Ben-Naim and Weitman, 2021). However, new races are continuing to emerge which evade these resistance mechanisms. Assessing the transcriptome of these downy mildew isolates has been essential in predicting secretome proteins which play an essential role in the colonization process (Guo et al., 2016). Despite this, there have been few nucleotide differences in identified effector proteins across our collected isolates. Therefore, investigation of other regions of the genome, as well as epigenetic modifications, is required to understand more of what causes these changes in pathogenicity.

Currently, the only way for growers to eliminate or prevent a basil downy mildew infection is to have used resistant cultivars, or try treating the basil seeds and plants with fungicides. Commercial basil growers often use a combination of different cultivars and fungicides to manage their operations (Gisi, 2002). However, this approach inadvertently leads to the development of downy mildew subpopulations which can colonize previously resistant plants, and/or are resistant to fungicides. By understanding more thoroughly the mechanisms by which this pathogen colonizes basil plants, future researchers will be able to take a more targeted approach to resistant cultivar and fungicide development.

Review of Literature

Sweet basil (*Ocimum basilicum*), is one of the most well-known herbs worldwide, noted for its unique smell, flavor, and appearance. Less known, but well documented, are the pharmacological properties of sweet basil, often used as an herbal supplement to reduce symptoms from fatigue, colds, spasms, and insect and snake bites (Ahmad Ch et al., 2015). Additionally, chemical derivates from sweet basil have been shown to have anticancer and antimicrobial properties (Ahmad Ch et al., 2015). These traits, combined with its myriad culinary uses have made basil a keystone of home and commercial gardening operations for generations.

Almost as ubiquitous as sweet basil to commercial growers, but sometimes overlooked by home gardeners, is basil downy mildew (*Peronospora belbahrii*). First reported in Uganda in 1933, but not formally described as a species until 2005, this oomycete plant pathogen causes grey-brown, furry growth on the underside of basil leaves as it colonizes its host (Belbahri et al., 2005). As an oomycete, basil downy mildew is an obligate, biotrophic pathogen, meaning that it

requires a living host. Chemical control of these downy mildews requires toxic fungicides, the frequent use of which promotes the emergence of resistant subpopulations, making the problem worse (Gisi, 2002). For this reason, many companies and research groups have begun to breed basil cultivars resistant to downy mildew infections.

In classifying a plants immune response, two different patterns must be discussed. First, pathogen associated molecular patterns (PAMPs), cause a PAMP-triggered immunity (PTI), response when detected by the host plant (Dodds and Rathjen, 2010). This type of immune response is often the result of the plant cell detecting specific molecules or metabolites in the extracellular space usually associated with the presence of specific pathogens (Dodds and Rathjen, 2010). The other immune response of interest is effector-triggered immunity (ETI), caused when the plant detects the presence of pathogen effector proteins in the apoplast or cytoplasm of its cells (Dodds and Rathjen, 2010). These effectors are small, secreted proteins used by the pathogen to aid in the colonization process.

Of this diverse family of proteins, two major categories emerge based on their localization: apoplastic, and cytoplasmic. Apoplastic effectors are secreted from the pathogen and localize to the space between the cell membrane and cell well in plant cells, while cytoplasmic effectors localize to the cytosol (Wawra et al., 2012). Oftentimes the localization of an effector can provide insight into its function. For example, apoplastic effectors generally mediate the invasion of host cells by helping to break down the cell wall, or inducing necrosis with toxins (Wawra et al., 2012). To determine localization and gain some insight into function, effectors are often expressed individually in host plants with fluorescent tags. Microscopic analysis then allows researchers to visualize their localization. Sometimes, these effectors play such a key role in the colonization process that they can induce disease symptoms when

expressed individually (Lopez et al., 2019). However, this is rare, and not always an effective way to gain insight into protein function.

In addition to structural homology and localization experiments, the presence of specific motifs in the coding regions of effector protein genes can provide significant insight into their role in host colonization. The most discussed of these motifs is the RXLR. The presence of this motif has been shown to help facilitate the entry of effector proteins into host cells, even without other pathogen machinery (Dou et al., 2008). That these effectors with the RXLR motif localize correctly even in the absence of the pathogen suggests that they may play a significant role in this process. However, some effector proteins which contain the RXLR motif in their coding region have this motif cleaved before secretion from the pathogen (Wawra et al., 2017). The reasoning for this difference is currently unknown but may relate to the pathogen adapting to prevent an ETI response. Understanding the role that these proteins have in the colonization process of sweet basil by *Peronospora belbahrii* will provide insight into strategies for developing cultivars resistant to currently observed isolates.

Alternatively, many research groups have turned their focus to understanding the immunity mechanisms of the host plant, instead of focusing on the pathogen secretome. One approach is to scrutinize the mechanisms by which the host first detects the presence of a colonizing pathogen. For example, the nucleotide-binding leucine-rich repeat receptor (NLR) ZAR1 in *Arabidopsis thaliana* detects the presence of the AvrAC effector protein in the cytosol (Wang et al., 2019). Effector detection triggers a kinase cascade which signals for the induction of ion flux across the plasma membrane as a component of the ETI response (Wang et al., 2019). The highly specific recognition of the AvrAC effector by the ZAR1 resistosome suggests that these plant-pathogen interactions are likely highly conserved.

While some researchers are interested in the specific mechanisms of host resistance, others study the development of resistant cultivars. Previously, the Pb1 gene in *O. basilicum* provided total resistance to basil downy mildew (Ben-Naim and Weitman, 2021). However, use of fungicides and resistant cultivars in commercial agricultural operations has spurred the development of *P. belbahrii* races which can overcome these established resistance mechanisms (Gisi, 2002). The development of resistant basil cultivars relies heavily on traditional Mendelian genetics to determine which genes may play a role in conferring this resistance. Researchers determined that the Pb2 gene in *O. basilicum* is incompletely dominant with Pb1, and both had to be present to provide resistance (Ben-Naim and Weitman, 2021). Applied genetic analysis such as this have allowed researchers to continue developing cultivars resistant to *P. belbahrii*, even as new races have continued to be reported. Without knowing how the pathogen is changing to adapt to these environments, however, it is difficult to be more targeted in cultivar development strategies.

A common method of identifying novel oomycete groups is through sequence analysis of their rDNA regions. The inter-transcribed spacer (ITS) regions lie between the 18s and 28s rDNA coding regions of the oomycete genome (Crawford et al., 1996). Due to their proximity to the highly conserved rDNA, these ITS regions are also themselves highly conserved within a species. However, slight variations in the ITS regions can imply emerging phylogenetic divergence, beyond what could be classified by morphology alone (Crawford et al., 1996). It was the amplification and sequencing of this region which led to the formal identification of *Peronospora belbahrii* in 2005 (Belbahri et al., 2005). Novel oomycete plant pathogens are generally first described by their ability to infect a specific host. In this case, basil plants affected

by downy mildew were observed, and thought to belong to an already described *Peronospora* species.

Since its identification over 15 years ago, *P. belbahrii* has continued to cause complications for basil growers worldwide for both citizen and commercial growers. Not only is management of these downy mildew infections difficult, but numerous reports from researchers and growers suggest that the population is changing to overcome the resistance mechanisms of basil cultivars more effectively. Some members of the population have even changed so much as to begin colonizing non-basil hosts (Rivera et al., 2016). ITS amplification and sequencing of a downy mildew infection on a group of coleus plants in 2015 showed 100% nucleotide identity with *P. belbahrii* (Rivera et al., 2016). These observations suggest a concerning amount of genetic diversity among the population of *P. belbahrii* isolates. While some of this diversity can be attributed to previous and ongoing use of chemical controls, it also provides insight into how the defenses of resistant cultivars have been so quickly overcome.

As compared to other oomycetes, and other members of the *Peronospora* genus in particular, *Peronospora belbahrii* has a relatively small secretome (Thines et al., 2020). This provides support for the claim that effector proteins are under high selection pressure, and often undergo positive selection as a given population diversifies (Dodds and Rathjen, 2010). Because oomycetes are obligate pathogens, it is difficult to work directly with the proteome, as contaminating plant material must be contended with. Instead, to study *P. belbahrii* with a wider lens than focusing on specific effectors, transcriptomic analyses are often used. This strategy relies on comparing the transcriptome of an infected and healthy basil plant to filter out the host associated transcripts (Guo et al., 2016). Then, as a reference genome for *P. belbahrii* was only published in 2019, these pathogen transcript reads were then pooled into a *de novo* assembly,

where eleven effector proteins of interest were identified based on presence of signal peptide, RXLR motif, and protein size (Guo et al., 2016). While studying these specific proteins can provide a great deal of insight to the underlying plant-pathogen mechanisms, they do little to answer our questions about population diversity overall. For this, we instead investigated the ITS regions of three isolates of interest and compared them to reference data from Thines *et al.* (2020) to gain a better understanding of how the *P. belbahrii* isolates used by our group compared to those from other sources.

Methods

P. belbahrii inoculation protocol

Upon reports of diseased basil plants, samples were collected from garden centers, greenhouses, and agricultural fields as whole plants or cut sections. After being received at the university, sporulation of *P. belbahrii* was forced by incubating overnight in a high humidity chamber, relative humidity ≥ 96%, with 10 hours of darkness (Cohen et al., 2017).

Sporulating leaves were collected from diseased plants, placed in sterile water, vortexed, and then the liquid filtered through cheesecloth to remove debris. The suspension was then filtered again through four layers of cheesecloth to remove sporangiophores, soil particulates, and other contaminating debris. The filtered suspension was then centrifuged at 5000 x g for 2 minutes, the supernatant removed, and the pellet resuspended in filtered, sterile water to a final concentration of 10^6 sporangia/mL as determined by hemocytometer count. These suspensions were then aliquoted, flash-frozen in liquid nitrogen, and stored at -80 ^{o}C for later DNA extraction. Additionally, suspensions of 10^4 sporangia/mL were prepared to inoculate 6–8-week-

old plants for cultivar trials or to replenish stock. The plants were sprayed until water ran off the leaves, and then incubating in a humidity chamber as described above. Only one *P. belbahrii* isolate was maintained in the greenhouse at a time to reduce the likelihood of cross-contamination.

Table 1. Background Information for *P. belbahrii* Isolates of Interest

Isolate ID	Initial Collection Location	Initial Host Basil Cultivar
Pb-KAMA-20	Worcester, MA	Genovese
Pb-LIHREC-21	Long Island, NY	Passion
Pb-HVO-21	Happy Valley Organics, South Deerfield, MA	Prospera

Cultivar Trial

A range of ten basil cultivars were selected to determine differential virulence of the *P. belbahrii* isolates of interest (Table 1, Fig. 1). Susceptible cultivars are as follows: 'Newton', 'diGenova', and '48104', an experimental Genovese-type cultivar susceptible to both basil downy mildew and *Fusarium* infection. Resistant cultivars used were: 'Mrihani', 'Devotion DMR', 'Obsession DMR', 'Passion DMR', 'Thunderstruck DMR', and different 'Prospera®' types. At 6-8 weeks of maturity, three individuals of each cultivar were inoculated with a *P. belbahrii* isolate using the protocol described above. Additionally, three individuals of each cultivar were "mock inoculated", using the same protocol but with only water. Disease incidence

(DI) was assessed as a percentage of true leaves demonstrating chlorosis and/or pathogen sporulation (Fig. 2).

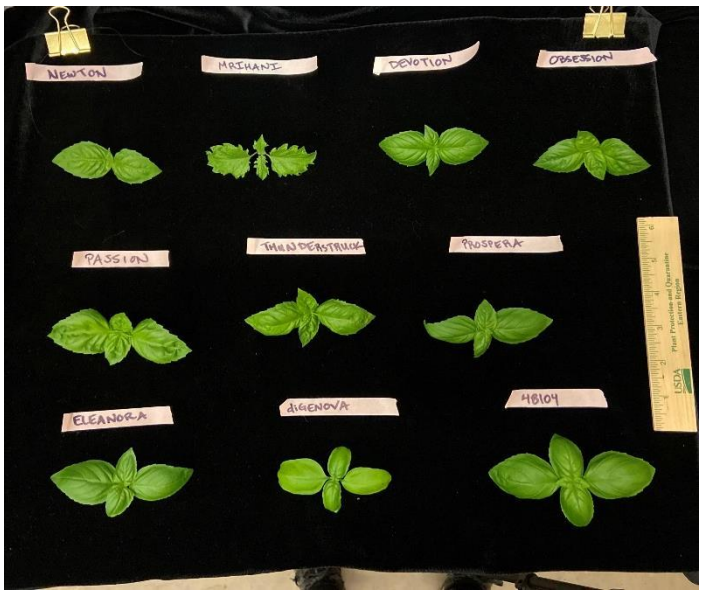

Figure 1. Sample leaves from each of the ten basil cultivars used for the cultivar panel. Represented are cultivars completely susceptible to basil downy mildew infection, as well those displaying different resistance mechanism.

DNA extraction

To extract genomic DNA (gDNA) from each of the three *P. belbahrii* isolates, a modified protocol from *Penouilh-Suzette et al., 2020* was used. Sporangia stored at -80 ºC was transferred to a Pink 1.5 mL homogenization tube for use in a BulletBlender, speed 10, for 30 seconds (Next Advance Inc.). Ground samples were then combined with 800 µL of lysis buffer preheated to 50 ºC (100 mM Tris-HCL pH 8, 10 mM EDTA pH 8, 1 M NaCl, 1% SDS) and 2 µL of proteinase K (Invitrogen, 20 mg/mL). Samples were incubated at 50 ºC for 30 minutes, mixing by inversion every 5 minutes. Then 270 µL of 5 M potassium acetate were added to the sample lysate, mixed by inversion, and incubated on ice for 10 minutes to precipitate non-DNA material. This precipitate was removed via centrifugation at 5000 g for 10 minutes at 4 ºC, with the supernatant being transferred to a new 2 mL tube. To digest contaminating RNA, 2 µL of RNAse A (Omega Bio-Tek Inc., 100 mg/mL) was added, mixed by inversion, and incubated at 37 ºC for 30 minutes. From this solution, the gDNA was precipitated by adding 1 mL of isopropanol, inverting 10 times, and incubating at room temperature for 30 minutes. Precipitated DNA was isolated via centrifugation at 10,000 g for 2 minutes, discarding the supernatant. The pellet was resuspended in 1 mL of 70% ethanol and collected by centrifugation at 10,000 g for 1 minute, discarding the

supernatant again. The gDNA was left to air dry for 15 minutes, and resuspended in 50 µL of TE buffer (10 mM Tris pH 8, 1 mM EDTA pH 8).

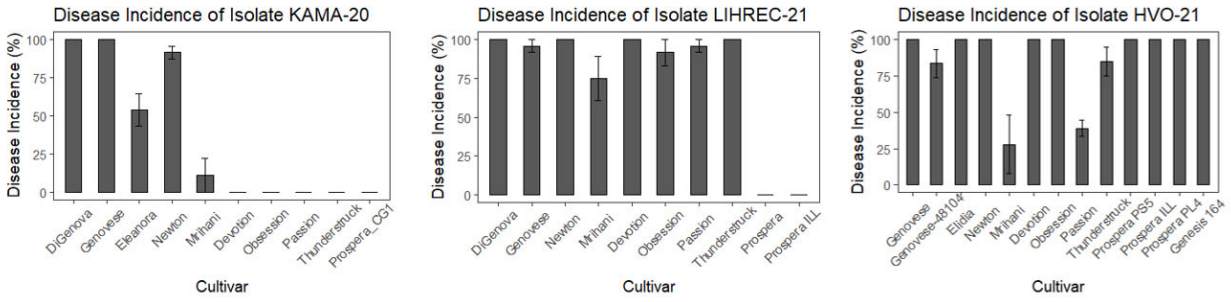

Figure 2. Each of the ten basil cultivars were inoculated with a P. belbahrii isolate of interest for this project. Disease incidence for each plant was rated as described above and averaged among the three individuals.

PCR amplification

Amplification of the ITS region was accomplished using a semi-nested approach with previously described primers universal to oomycetes (Table 2, Fig. 3). The first reaction mixture contained 1 x Q5 Reaction Buffer, 200 µM dNTPs, 0.5 µM of DC6 and ITS4 primers, 0.005 U of Q5 DNA Polymerase (New England Biolabs), and 100 ng of gDNA for a total reaction volume of 25 µL. These amplifications were performed using a Vapo Protect Master Gradient thermocycler (Eppendorf) using the following protocol: initial denaturation of 96 ºC for four minutes followed by 25 cycles of denaturation for 30 seconds at 96 ºC, annealing for 30 seconds at 50 ºC and extension for 1 minute at 72 ºC. Final extension was performed for 10 minutes at 72

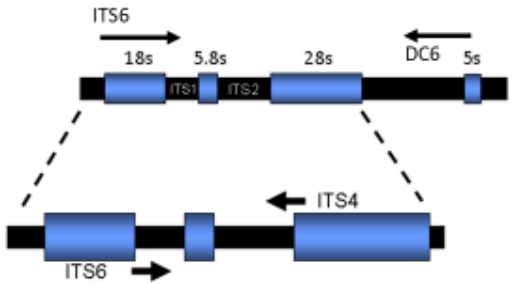

Figure 3. Adapted from Grünwald et al. 2013. This gene map shows the relevant rDNA of the oomycete genome. The top map shows the region amplified during the first round of PCR, while the bottom section shows the more specific region amplified during the second round.

°C. Products were separated using 1% agarose gel in 1 x TAE for 45 minutes at 120 V, and stained with SYBR Safe (ThermoFisher Scientific) (Fig. 4).

Table 2. Primer Sequences Used for Amplification of Oomycete ITS Regions

Primer	Sense	Primer sequence (5' → 3')	Location in rDNA
DC6	Forward	GAGGGACTTTTGGGTAATCA	18S gene
ITS6	Forward	GAAGGTGAAGTCGTAACAAGG	18S gene
ITS4	Reverse	TCCTCCGCTTATTGATATGC	28S gene

The second amplification was accomplished using the same reaction mix as the previous amplification but using the ITS6 primer instead of DC6. The cycling conditions were also the same, and the template DNA was 1 µL of the product from the previous reaction. These products were visualized using the same gel electrophoresis conditions as the first reaction (Fig. 4).

Figure 4. PCR amplification of the ITS regions of the P. belbahrii genome and portions of the surrounding 18S and 28S rDNA genes visualized on 1% agarose gel. **A.** The first PCR amplification of the semi-nested protocols using DC6 and ITS6 primers. Lane 1 contained the 1kb plus GeneRuler ladder (New England Biolabs), lanes 2-4 contained amplicons from isolates KAMA-20, LIHREC-21, and HVO-21, respectively, but were unable to be visualized. Lane 5 contained a product from a Fusarium oxysporum used as a positive control. **B.** The second PCR amplification using the products from (A) as a template. Lane 1 contained the same molecular weight marker as (A). Lanes 2-5 contained amplicons in the same arrangement as (A), and all were visualized successfully.

Table 3. *P. belbahrii* gDNA Quantity and Quality

Sample ID	DNA Concentration (ng/µL)	260/280	260/230
KAMA-20	465	2.07	2.76
HVO-21	116	2.00	4.52
LIHREC-21	570	1.90	1.89

Phylogenetic analysis

PCR amplicons from the second step of the semi-nested protocol were purified using the GeneJET PCR Purification Kit (ThermoFisher), and each product sent for forward and reverse Sanger sequencing via Azenta Life Sciences. For each isolate, these forward and reverse sequences were combined into a consensus read using Benchling. These consensus sequences were then manually aligned to other ITS amplicons from *Peronospora* isolates characterized by Thines *et al.* (2020). All 30 sequences were uploaded to MEGA 11 to be assembled into a phylogenetic tree using the neighbor-joining bootstrap algorithm with 1000 replications (Fig. 5).

Results

In recent years collaborators and commercial growers have reported basil downy mildew isolates overcoming resistant basil cultivars. These observations were the impetus for this project. Comparing ten basil cultivars to three *P. belbahrii* isolates, we observed three different pathogenic phenotypes (Table 3, Fig. 2). These three *P. belbahrii* isolates were each collected

from different locations and growers during 2020/2021 (Table 1). The isolate KAMA-20 was unable to significantly overcome any of the resistant cultivars, while HVO-21 successfully colonized each of the ten basil cultivars compared. Intermediately, the LIHREC-21 isolate colonized some resistant cultivars, but did not successfully infect 'Thunderstruck', 'Prospera', or 'Passion ILL' plants (Fig. 2).

After completion of the cultivar panel, each of the three pathogen isolates were isolated from their cultivar of origin and stored for DNA extraction. The quantity and quality of the extracted gDNA was analyzed using a Nanodrop spectrophotometer (ThermoFisher) (Table 3). While these absorption values suggest room for improvement in the DNA extraction protocol, the samples were sufficient for ITS PCR. Additionally, the gDNA was visualized on 1% agarose gel to inspect shearing and molecular weight. We observed that while the DNA was significantly fragmented, resulting in smeared lanes, the bulk of the gDNA had a molecular weight in the range of 10 kb (Fig. 6).

Extracted gDNA from each isolate was used as the template for the first PCR amplification using DC6 and ITS6 primers. The amplicons for the three oomycete isolates were unable to be visualized, but the amplicon from *F. oxysporum* gDNA used as a control showed a strong band in the range of 700 bp (Fig. 4). These amplicons were used as the template DNA for the second sound of PCR using the ITS4 and ITS6 primers. Each were successfully visualized on 1% agarose gel, in the range of 700 bp, the expected molecular weight (Fig. 4). The fifth lane, from the *F. oxysporum* sample, shows multiple bands (Fig. 4).

Once amplicons were purified and sequencing results received, they were aligned and assembled into a phylogenetic tree with ITS data from Thines *et al.* (2020) (Fig. 5). The three isolates described in Table 1 are highlighted in red. From this diagram, we observe that each

isolates differential pathogenicity is reflected in their ITS genetic differences (Fig. 2, 5). LIHREC-21 and HVO-21 isolates are close neighbors to different *P. belbahrii* collected from sweet basil, while KAMA-20 was closes to *P. elsholtziae*, isolated from *Elsholtzia splendens*, a genus in the same *Lamiacae* family as sweet basil (Fig. 5).

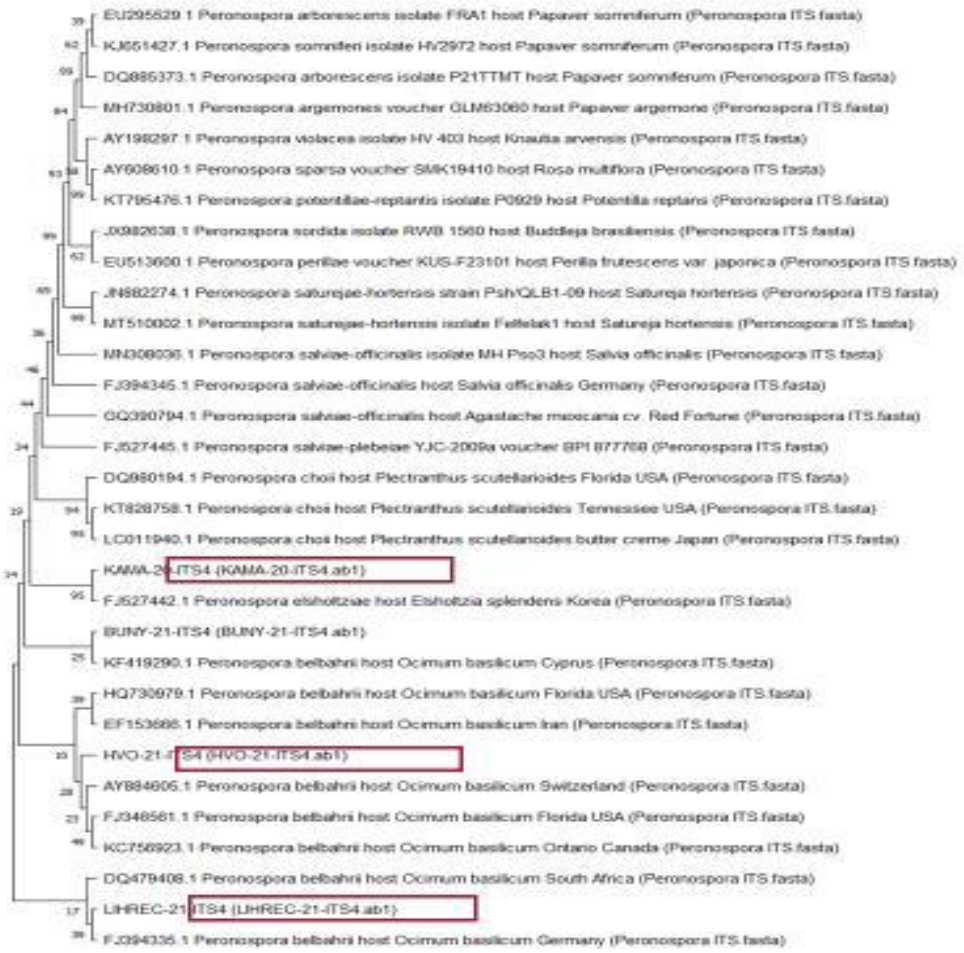

Figure 5. This phylogenetic tree was assembled using MEGA 11 with a neighbor-joining bootstrap algorithm. Isolated highlighted in red represent the three isolates of interest. Other sequence data was sourced from Thines et al. (2020) as comparison.

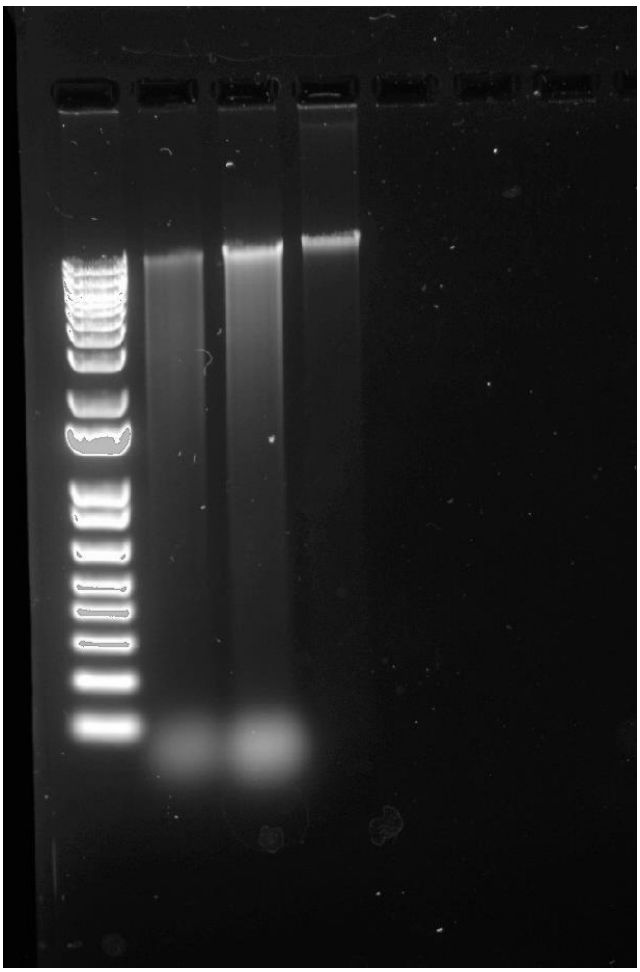

Figure 6. 1% agarose gel used to visualize the extracted gDNA from the three P. belbahrii isolates of interest. Lane 1 contains a 1 kb plus GeneRuler molecular weight marker, and lanes 2-4 contain the gDNA from KAMA-20, LIHREC-21, and HVO-21 respectfully.

Discussion and Future Directions

An open question in the study of downy mildew pathogens is the emergence of new races. Typically, these races are defined by their ability to differentially colonize resistant host cultivars (Crawford et al., 1996). However, recent literature has demonstrated that these emerging races may also be identified by comparing the highly conserved ITS regions of the

oomycete genome (Thines et al., 2020). In this experiment we demonstrate that a semi-nested PCR approach allows for the successful amplification of these regions, using primers originally designed for fungal isolates (Fig. 3, 4). As the ITS region is so small, even slight changes in sequence identity may cause drastic differences when compared using a neighbor-joining algorithm (Fig. 3). The small size and high conservation of these ITS regions allows them to be used as a proxy for overall genetic diversity in a population (Voglmayr, 2003). Much like using the 16S rDNA of bacteria, species and racial identification of *Peronospora* can be accomplished with ITS comparisons.

Understanding the underlying genetic variance that corresponds to observed pathogenic phenotypes can provide important insight to overall population changes among *P. belbahrii.* Comparing the pathogenicity of three isolates across ten basil cultivars showed three distinct phenotypes (Fig. 2). Of interest is the apparent binary nature of DI, most cultivars were either completely resistant, or showed disease symptoms on all leaves (Fig. 2). This is likely due to the presence or absence of a relevant resistance gene, and the airborne nature of the pathogen (Wyenandt et al., 2015). These cultivar panels allow us to visualize different pathogen phenotypes, but do not provide insight into reasons for these differences. For that understanding, we must investigate the overall genetic differences among pathogen population samples.

Building a phylogenetic tree using a neighbor-joining bootstrap algorithm allows us to visualize the differences in ITS sequence identity between our collected isolates, and data published by Thines *et al.* (2020). We observe that the phenotypic differences shown by the cultivar panel are reflected in population phylogeny (Fig. 5). This suggests the future potential of ITS sequencing as a diagnostic tool for basil downy mildew infections.

As current environmental and fungicidal controls for basil downy mildew are both costly and ineffective, resistant basil cultivars remain the best solution to combatting this pathogen (Gisi, 2002). If we are able to use genomic sequencing to predict which basil cultivars will be resistant/susceptible to specific *P. belbahrii* isolates, growers will be able to better prepare. For example, a greenhouse infection with high sequence similarity to LIHREC-21 would be best combatted with the purchase of 'Prospera' basil cultivars. Additionally, collection of diverse *P. belbahrii* isolates, and their sequenced ITS regions, will provide insight into the population divergence of this downy mildew. Using the ITS regions as a proxy for genetic diversity, we will be able to understand the rate at which this pathogen's genome is changing, and eventually identify which genes are conferring the ability to overcome these resistance mechanisms.

Due to the close link between plant and pathogen, management of basil downy mildew remains an ongoing struggle. These studies are the first steps to understanding what is happening to *P. belbahrii* at the population level, data which we hope to later connect to the development of new resistant basil cultivars.

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