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## Towards a Molecular Method for the Detection of Leaf Rust in Lowbush Blueberry

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TOWARDS A MOLECULAR METHOD FOR THE DETECTION OF LEAF RUST IN  
LOWBUSH BLUEBERRY

by

Steven Valentino

A Thesis Submitted in Partial Fulfillment  
of the Requirements for a Degree with Honors  
(Molecular and Cellular Biology)

The Honors College

University of Maine

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Advisory Committee:

Seanna Annis, Advisor, Associate Professor of Mycology, Associate Extension Professor

Sally Molloy, Assistant Professor of Genomics, Department of Molecular and Biomedical Sciences, University of Maine Honors College

Chris Mares, Director, Intensive English Institute,

Jianjun Hao, Assistant Professor of Plant Pathology, School of Food and Agriculture

David Yarborough, Wild Blueberry Specialist, Professor of Horticulture, University of Maine

# Abstract

*Thekopsora minima*, or leaf rust, is a fungal pathogen that infects *Vaccinium angustifolium* (lowbush blueberry), an economically important crop to the state of Maine. *T. minima* undergoes a complicated life cycle that contains five unique spore stages. It causes abscissions in the leaves of plants that may consequently lower yields in the next growing cycle if leaf drop is severe. Currently, growers are instructed to apply fungicides in late July to prevent further infection. However, this is often not effective due to poor timing. Data on spore release patterns would be beneficial to elucidate the infection period. However, microscopic identification of *T. minima* spores based on visualization of morphology alone is complicated. Therefore, prediction of major infection periods must be achieved by some other means to quickly identify the pathogen. In this regard, molecular methods involving the polymerase chain reaction (PCR) have potential as a solution. In this study, leaf samples that were suspected to be infected with rust fungus were collected, spores harvested, and DNA extracted from the gathered spores. Several different methods were utilized for this purpose and their efficacy is discussed. After amplification with universal fungal primers, DNA was confirmed to be from *T. minima* with 99 % identity to previously discovered sequences from highbush blueberry and confirm previous studies that reported a similar sequence from the same field site. Alignments of these data to similar species provided several sections of sequence unique to leaf rust that could serve as an aid in developing primers.

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# Table of Contents

Introduction.....	1
Materials and Methods.....	9
<i>Sample Collection</i> .....	9
<i>DNA Extraction</i> .....	10
<i>DNA Quantification</i> .....	10
<i>DNA Precipitation</i> .....	11
<i>Polymerase Chain Reaction</i> .....	11
<i>TBE Agarose Gel Excision of DNA</i> .....	12
<i>DNA Sequencing</i> .....	13
<i>Sequence Analysis</i> .....	13
Results.....	13
Discussion.....	23
Literature Cited.....	29
Author's Biography.....	32

## List of Figures and Tables

<b>Figure 1:</b> Underside of Blueberry Leaf Infected with <i>T. minima</i> , displaying Uredinia....	4
<b>Figure 2:</b> Uredinospores from <i>T. minima</i> on a Section of Spore Trap Tape.....	6
<b>Table 1:</b> Components to Make a 20 µl PCR Reaction.....	12
<b>Table 2:</b> DNA Quality from <i>T. minima</i> spore samples obtained with the Vacuum Method of spore collection.....	17
<b>Figure 3:</b> DNA Extracted from <i>T. minima</i> spores collected from Infected Leaves through Multiple Methods.....	17
<b>Table 3:</b> DNA Quality from <i>T. minima</i> spore samples obtained with the Wash Method of spore collection .....	18
<b>Figure 4:</b> DNA concentrated from vacuum collected spores and washed leaf samples...	18
<b>Table 4:</b> DNA Quality Data from Samples after Precipitation.....	19
<b>Table 5:</b> DNA Quality from spore samples obtained with the Modified Vacuum Method and extracted by multiple methods.....	19
<b>Figure 5:</b> PCR with DNA Extracted from Putative Leaf Rust Samples 16 and 17.....	20
<b>Figure 6:</b> PCR with DNA Extracted from Putative Leaf Rust Sample 20.....	21
<b>Table 6:</b> Possible Sequences Unique to <i>T. minima</i> Compared to Other Rusts.....	21
<b>Figure 7:</b> Alignment of Putative <i>T. minima</i> DNA against ITS Regions of Other Rust Samples.....	22

# Introduction

Lowbush blueberry, *Vaccinium angustifolium*, is an important crop for the Maine agricultural economy. Indeed, Maine is one of the largest producers of blueberries in the world, growing around 10 percent of all blueberries in North America (Yarborough, 2015). The downeast region of the state is where the majority of these plants are distributed, and where they are grown in barrens. *V. angustifolium* is one of about 450 *Vaccinium spp.* thought to exist worldwide, with the most genetically diverse crops being located in North America (Trehane, 2004). The plant is managed with a two year, crop-prune cycle, with a specific field bearing fruit every other year. This system is designed so that half of the fields are encouraged to vegetatively grow and the other half is harvested at the end of the cycle in August. In the past, yields averaged around 7,700 kg/ha, but ranged greatly, from 300 to 17,000 kg/ha (Hepler and Yarborough, 1991).

Attempts at the blueberry's domestication have not been met with great success due to slow establishment and inefficient rhizome production (Yarborough, 2012). Thus, cultivation of the blueberry crop is a slow and arduous process such that all of the current harvest is based on wild plants. Since it is extremely difficult to introduce disease-resistant plants, growers must rely on alternative methods for the containment of various diseases among the wild crop. Fungicides, as well as the burning of crops for pruning purposes, are often used as a pest management tool to control the spread of these pests (Annis and Yarborough, 2015).

One phytopathogen of lowbush blueberry in Maine is *Thekopsora minima*, a rust fungus which primarily infects the leaves. It is also found on lowbush blueberry in

various other locations around the world, including but not limited to, Canada, New York (Sato et al., 1993) and Japan (Kobayashi, 2007). The disease is known as blueberry leaf rust, one of many diseases known to be caused by rust fungi. These pathogens are from the order *Pucciniales* (Aime et al., 2006). There are approximately 7,000 species of rust in the world and many affect important crops such as wheat and coffee (Mohan, 2010). Rust fungi follow a highly complex life cycle that consists of up to five distinct stages, with each stage contributing to a different part of the overall disease process. Indeed, the taxonomical classification of this order of fungi is based upon the spore types and life cycle found in a particular species (Petersen, 1974).

Another unique feature of the rust fungi is their obligate biotrophic nature, which means that they rely on a living host for their survival (Cummins and Hiratsuka, 2003). As they require their host to survive, they don't kill their host. Instead, they have evolved other adaptations that allow for its survival within the plant species, such as proteins required for nutrient assimilation and loss of polysaccharide-degrading enzymes (Duplessis, 2011). Interestingly, they also establish structures called haustoria. Haustoria are hyphal tips that penetrate the host cell wall, allowing it to draw key nutrients from the space between the plant cell wall and plant plasma membrane without actually disrupting the membrane. This causes a major invagination in the plant plasma membrane that appears to enable the fungus to release signals that affect the plant's immune system. For instance, flax rust secrete avirulence proteins that are recognized by host resistance factors, leading to a compatible association (Catanzariti et al., 2006). Regardless, long term systemic infections can ultimately lead to reduced crop yields. This is mainly due to

a lack of surface area for the plant to capture light and perform photosynthesis efficiently (Agrios, 2005).

Adding one more degree of difficulty to their study, rusts are usually heteroecious, using phylogenetically unrelated hosts to complete their life cycle (Cummins and Hiratsuka, 2003). This further complication of the life cycle makes it difficult to control their spread, as there is an additional component to consider. This particular rust, *T. minima*, uses *Tsuga* spp. (hemlock) as the alternative host to complete part of its cycle before infecting *V. angustifolium* (lowbush blueberry) to complete the other part (Sato et al., 1993). Being a macrocytic rust, *T. minima* also has a life cycle that contains all five of the possible stages of rust spores, including a repeating stage with uredinospores on blueberry (Cummins and Hiratsuka, 2003).

The disease process begins when aeciospores infect blueberry leaves in the summer. Structures called uredinia then begin to develop on the lower surface of the leaf, harboring many uredinospores within them. These spores represent the repeating stage of the cycle in which they can continuously re-infect *V. angustifolium*. New pustules can develop on the other leaves and produce even more uredinospores, which explains how the infection can be spread during this stage of the cycle. Millions upon millions of these microscopic spores are released, and are quite easily transported by wind (DAFWA). This process continues until late fall, when the fungus produces telia on the leaves, which can overwinter on the ground. Teliospores eventually germinate and produce basidiospores in the early spring. The hemlock tree, the alternative host, is infected by these basidiospores, where the fungus continues its life cycle until it infects *V.*

*angustifolium* with aeciospores in the spring. Thus, the disease can reappear in a particular location during the following growing season in the spring.

Blueberry leaves initially infected with leaf rust will exhibit yellowish spots on the upper surface of young leaves. Later on, the spots become reddish and often larger than those present during initial infection. Many times, anthocyanescence, or the reddening of the leaf, is seen during the progression of disease (Nelson, 2008). Powdery yellowish pustules on the underside also appear, indicating a large amount of uredinia across the leaf's surface area (Fig. 1). In Hawaii, severity of the disease is associated with wet, moist conditions (Nelson, 2008). In the more severe cases, a browning of the leaf, accompanied by premature leaf drop, is observed, depriving the plant of its photosynthetic capabilities. In Hawaii, blueberry rust represents the most serious disease facing the native blueberry crop (Nelson, 2008).



**Figure 1.** Underside of Blueberry Leaf Infected with *T. minima*, displaying Uredinia. Pustules are yellowish-orange in color. Photo by: Annis Lab

Currently used disease management practices rely on fungicides because other external control measures, such as removing nearby hemlock trees in the area would not

prove successful (Annis and Yarborough, 2015). One explanation for this phenomenon is the fact that rust spores can travel for miles by wind, allowing them to infect distantly located leaves. As an additional control measure, growers could use disease-resistant plants for cultivation. However, as mentioned previously, this is not easily accomplished due to slow establishment of the crop. As these plants are generally unavailable, other attempts are made to prevent genetically novel forms of the pathogen from being introduced into a particular area. In Australia, for example, the pathogen is deemed a serious list A quarantine pest (Bishop, 1997). Fungicide application is usually ineffective, though, because of a lack of understanding of the temporal pattern of disease. Therefore, alternative approaches should be attempted to elucidate such patterns.

Since the spores of the rust fungi are quite difficult to differentiate based on visualization of morphology alone, it is believed that nucleotide differences between fungal species could potentially be used to identify a particular species in a sample. An example of uredinospores from this rust fungus can be seen in Figure 2. The uredinospores are about 20  $\mu\text{m}$  in diameter, are oval in shape, and look like many uredinospores of other rust species, which presents a problem. Indeed, there are many other rust species that have this type of uredinospore. It is thus difficult to gather data from spore traps and discover new information about this disease as there is currently no way to distinguish the uredinospores from those of similar species. The use of molecular biological techniques is one way that researchers are hoping to solve this dilemma. By using probes that recognize specific DNA sequences in the *T. minima* genome, the pathogen can be correctly identified. If timing and environmental conditions related to

the spore dispersal of the pathogen could then be elucidated using these methods, these data could then be disseminated to growers in order for them to better manage their crops.



**Figure 2.** Uredinospores from *T. minima* on a Section of Spore Trap Tape. Spores are roughly 20 μm in diameter, oval, and similar in appearance to those of other rust fungi. Photo by: Annis Lab

The polymerase chain reaction, or PCR, is a simple molecular biological technique that is used to amplify many copies of a sequence of DNA from a single copy. It uses a heat-stable DNA polymerase from a thermophilic bacteria to create new DNA from short nucleotide primers specific to a particular section in the genome (Mullis and Faloona, 1987). The highly variable ITS region is one of the most commonly used regions for the amplification of fungal DNA (Gardes et al, 1991). With a majority of the variation occurring in the two non-coding sequences nested in between the conserved small ribosomal subunit 18S, 5.8S, and large subunit 28S rRNA genes, it is ideal for the molecular identification of fungi. In addition to initial “universal” primers (ITS1 and ITS4), primers specifically targeting higher fungi (ITS1-F) and Basidiomycota (ITS4-B) have been created (Gardes et al, 1991 Gardes and Bruns, 1993). The standard use of these

primers in laboratories makes them promising candidates for species identification, especially when one cannot rely on morphological observations alone to come to a conclusion.

In previous experiments, certain applications and variations of the polymerase chain reaction (PCR) have been successful in identifying certain species of fungi from airborne spores and providing a means for their quantification (Rogers et al., 2009, Fountaine et al., 2010). These applications have some key features that provide scientists with important information about fungal plant disease. First, they find unique, species-specific regions of fungal DNA whose amounts can be quantified through amplification with primers. Secondly, the level of this specific DNA region tends to be directly correlated with the amount of fungus present in an individual plant or an entire crop region. Thus, taken together with various weather patterns in the area, and the physiological state of the plant, predictions about the spread and timing of disease can be made. For example, Fountaine et al. (2010) were able to exclude the airborne inoculum of *Rhynchosporium secalis* spores from being the primary cause of leaf blotch infection, which was propagated instead from seeds that had been infected with the pathogen. Another advantage that molecular detection methods have is that they can sometimes provide a qualitative assessment of the disease in an area faster than traditional means, resulting in higher throughput of assays (Margarey et al. 2008).

With regards to its applications to rust species in particular, the universal ITS primers (White et al., 1990) were shown to amplify fungal DNA at the exclusion of DNA from the host plant species (Gardes and Bruns, 1993). Restriction Fragment Length Polymorphism (RFLP) patterns detected in multiple infected plant species were shown to

match those found in rust spores alone, even when there was a high likelihood that plant DNA served as the predominant template in the reactions. This means that methods involving molecular biology can be formulated to be able to discriminate against plant nucleic acid and select for a certain fungal species instead. Indeed, this finding has broad implications for plant pathology. It is an indication that there is a hypothetical ability to discover a certain species in any given plant without relying on the experience of a trained specialist. In other words, a great deal of information can be accumulated regarding the composition of a plant solely based on these sorts of molecular techniques.

Given the inefficiency and inaccuracy involved with relying on morphology and light microscopy for analysis of some diseases in a given crop, it becomes clear that there is a need for a new, more rapid method of detection of the rust fungus of lowbush blueberry. A molecular detection method could therefore present an effective solution to this dilemma. Data obtained using PCR with DNA harvested from an infected crop has the potential to provide crucial insight into the extent of disease in a given field, as well as the timing of spore release. Also, with the development of such a procedure, the chances of correctly identifying spores as *T. minima* greatly increase. This work presented here seeks to expand upon our current knowledge of the rust fungus and contribute towards the creation of a method that delivers data on blueberry leaf rust samples quickly, reliably, and accurately. With these data, growers can monitor high-risk fields and weather patterns and use the gathered information to eliminate the disease from their crops.

## Materials and Methods

**Sample collection.** Two alternative methods were employed to collect spores of the leaf rust fungus. The first method utilized a vacuum that was modified to capture spores in a pipette tip at the end (Helfer, 1985). In this method, infected leaves were placed under a dissecting microscope and examined for rust pustules. A vacuum line was prepared with clear tubing, and connected using Parafilm (Kimtech Science, Roswell, GA). After being washed with 0.05% Tween 20, a 1000  $\mu$ l pipette tip was added to the end. In later preparations, the technique was modified so that the tip was cut with a razor roughly 3 cm from the large end in order to decrease flow velocity without greatly affecting vacuum pressure. A Kimwipe was placed in between the tubing and the pipette in order to collect spores that might escape. Once construction was complete, the vacuum was fully turned on and the tip held over heavily infected areas of the leaf in order to obtain spores. For each sample, roughly 30-40 leaves were sampled. Samples were washed from the pipette tip with 0.05 % Tween 20 into a 1.5 ml microcentrifuge tube. The pipette tip was then cut up with a razor, the pieces inserted into several micro-centrifuge tubes, and spun down to remove any remaining spores. Spores from all tubes were finally consolidated into one spore suspension and stored at -20°C.

The second method was adapted from a procedure for the collection of powdery mildew cleistothecia from blueberry leaves (Pearson and Gadoury, 1987). First, leaves were removed from the stems and placed into a 1 L Erlenmeyer flask. Distilled water was then added so that all leaves were covered and the mixture shaken for 10 minutes at around 200 RPM on a platform shaker. The solution was passed through a colander and 50 ml of the flow-through was added to centrifuge tubes. Samples were centrifuged at

10,000 RPM for 10 minutes and the supernatant removed. All samples were stored at -20°C until needed for further experimentation.

**DNA Extraction.** All spore samples were pelleted using a microcentrifuge so that any of the remaining Tween 20 in the tubes could be removed. One of two methods of spore disruption were then employed; bead beating or freezing with liquid nitrogen. Most of the spores obtained through vacuum collection were subjected to the bead beating method, while those harvested by washing were subjected to the liquid nitrogen method. For the bead beating method, 400 µl of SGF1 buffer (Omega, Tarzana, CA) was added and the pelleted spores were transferred to 2 ml bead-beater tubes and ground by 0.5 mm beads for three minutes through vortexing. In the second method, samples were snap-frozen in liquid nitrogen and ground with a pestle in a microcentrifuge tube. Some of the later collections of spores obtained through the modified vacuum method went through a cold/heat shock by being placed in -80° C for 30 minutes and then heated at 65° C for one hour before the bead beating step. Following both procedures, the E.N.Z.A. SP Fungal DNA Mini Kit (Omega, Tarzana, CA) protocol for fresh/frozen specimens was used, along with a slight modification at the final elution step. Rather than using the recommended 100 µl elution buffer volume, two volumes of 40 µl were used. In later procedures, total elution volume was changed to 50 µl.

**DNA Quantification.** A spectrophotometer (NanoDrop 2000, Thermo Scientific, Rochester, NY) was used to initially analyze the DNA concentration and calculate the  $A_{260}/A_{280}$  ratio for a preliminary assessment of purity. To confirm these results, gel electrophoresis was routinely performed for the visualization of band intensity. Gels were made of 0.8% agarose (SeaKem LE) and 30 ml of 1X Tris Borate EDTA (TBE) with 3µl

of GelStar staining dye (Lonza, Basel, CH) added. 1 µl of Lambda/HindIII ladder was used for band reference and approximately 2 µl of each sample and 2 µl of loading dye (New England BioLabs, Ipswich, MA) was added.

**DNA Precipitation.** Due to initially low yields of nucleic acid, DNA collected early in the process was pooled together. After making a separate batch for each collection method (vacuum and wash), half of the total volume of 7.5 M ammonium acetate was added. Total volume of sample and ammonium acetate was calculated and an equivalent amount of isopropanol was added before incubating on ice for 30 minutes. After incubation, samples were spun down for 10 minutes at full speed in a microcentrifuge. Supernatant was removed and the pellet was washed with 500 µl of 70 % ethanol. Solution was spun for one minute for repelleting before removing the ethanol and drying. Samples were then suspended in 40 µl of 10 mM Tris and stored at -20° C.

**Polymerase Chain Reaction.** PCR reactions were set up with a total volume of 20 µl and components in the proportions listed in Table 1. Negative controls contained all components of the PCR master mix and 5 µl of sterile Milli-Q water substituted for DNA. Positive controls contained all components of the PCR master mix and 5 µl of *Valdensia* DNA substituted for sample DNA. Thermocycler (Amplifon II Thermolyne) parameters were as follows: predwell denaturing at 94°C for 5 mins, and then 30 cycles of a denaturing step at 95°C for 1 min, annealing temperature at 55°C for 1 min, and an extension period at 72°C for 1 min, followed by a postdwell at 72°C for 10 mins, and an indefinite chill period at 4°C (Jasalavich et al, 2000).

**Table 1.** Components to Make a 20  $\mu$ l PCR Reaction

<b>Component</b>	<b>1X Vol for Master Mix (<math>\mu</math>l)</b>
5X GoTaq Buffer	4
25mM MgCl <sub>2</sub>	1.2
1.25uM dNTPs	3.2
10uM ITS1 Primer	1
10uM ITS4 Primer	1
Gotaq Polymerase	0.2
DNA Sample	Up to 5
Sterile Milli-Q Water	Variable to make up to 20 $\mu$ l Total

To visualize amplification results, gels composed of 1.2% agarose (SeaKem LE) and 30  $\mu$ l of 1X Tris Borate EDTA (TBE) were made. 3  $\mu$ l of GelStar was added to the agarose solution before pouring into the gel apparatus. 0.5  $\mu$ l of 100 base pair ladder (New England BioLabs, Ipswich, MA) was used for size reference standard. Approximately 5  $\mu$ l of each PCR sample was loaded with a ~2  $\mu$ l of 6X Blue/Orange loading dye (New England BioLabs, Ipswich, MA), and run at 100V.

**TBE Agarose Gel Excision of DNA.** In cases where two bands were present, both bands were excised from the gel using a sharp blade and each placed into a microcentrifuge tube. The QIAEX II Gel Extraction Kit (Qiagen, Alameda, CA) was then used to bind the DNA, wash away impurities, and prepare it for sequencing.

**DNA Sequencing.** The microcentrifuge method of the QiaQuick PCR Purification Kit (Promega, Fitchburg, WI) was used to rid selected samples of impurities and make the nucleic acid sample suitable for sequencing. NanoDrop was used to confirm concentration of DNA. Samples were sent to the University of Maine DNA Sequencing Facility (<http://www2.umaine.edu/dnaseq/>) and sequenced using the ITS1 and ITS4 primers (White et al, 1990) as the forward and reverse reactions, respectively.

**Sequence Analysis.** BioEdit (Hall, 2013) was used to create a contiguous sequence from the forward and reverse sequences, and sequence inconsistencies were resolved based on chromatogram results. The final consensus sequences were then input into the Basic Local Alignment Search Tool (BLAST, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), Altschul et al., 1990) against previously sequenced *T. minima* samples within the GenBank nucleotide database (Burks et al., 1991) for potential matches. If DNA was confirmed to be from *T. minima*, data was then compared to other top BLAST hits using a multiple sequence alignment accessory program (Clustal W) in BioEdit.

## Results

The first spore collection method employed, the vacuum line, did not appear to be successful at collecting a sufficient number of spores for the successful extraction of DNA using the bead beating method. DNA extracted using the bead beating method with samples 5, 6 and 7 was minimal in concentration and DNA extracted from all samples (1-7) had  $A_{260}/A_{280}$  ratios outside of the ideal 1.8-2.0 range (Table 2). Furthermore, none of these samples (1-7) had DNA that was visible on a gel (Figure 3), confirming that there were very low overall amounts of DNA. In the samples of spores collected with the

second collection method (wash method),  $A_{260}/A_{280}$  ratios observed were consistently lower than the ideal 1.8-2 range (Table 3), indicating that the quality of the DNA extracted was quite low overall. The subpar quality was also demonstrated by the gel, which did not show bands for samples 9, 12, or 13 (Figure 3). In addition, PCR performed on this particular collection of samples didn't amplify DNA using the universal fungal primers ITS1 and ITS4 (data not shown).

Unable to collect large amounts of DNA of sufficient quality with these two methods, samples of previously extracted DNA were precipitated together, creating a higher concentration of DNA in the precipitates (Table 4, Figure 4) but with  $A_{260}/A_{280}$  ratios that were still low (Table 4). The precipitated wash sample (PrecipWash) failed to consistently amplify using the universal fungal primers ITS1 and ITS4, after showing promising results initially. Multiple PCR attempts failed to produce an amplicon, after initially amplifying. The precipitated vacuum sample (PrecipVac) did not produce an amplicon at all.

The last spore collection method employed was a simple modification of the vacuum method achieved by cutting a small portion of the pipette tip to decrease the fluid velocity during the vacuuming procedure. Spore samples obtained through this procedure did not have an increased DNA yield upon extraction but exhibited higher DNA quality based on  $A_{260}/A_{280}$  ratios close to or within the ideal range of 1.8-2 (Table 5). It was DNA samples from spores collected with this procedure that were achieved amplification with PCR.

PCR using DNA from samples 16, 17, and 20 (Table 5) produced amplicons even though band strength was weak (Figure 5 and 6). The amplified products of samples 16

and 17 were, based on prior experiments (Horton, 2015), not quite as large as expected, since they were only around 500 bp in length instead of 700 bp (Figure 5). Each amplified DNA fragment was purified and both strands were sequenced, after which consensus sequences were constructed from the resultant chromatograms. For sample 20, two amplification products were seen, around 450 bp and 700 bp respectively (Figure 6). Since the bands were spaced far enough apart on the gel, each individual band was cut and the resulting DNA purified and sent for sequencing. Based on chromatogram results, a consensus sequence could be made for one band (700 bp) but not the other (450 bp), the latter displaying many indeterminate peaks.

BLAST was used to match the sequences of amplified bands to previously submitted sequences in the GenBank database. Input of the sequence data obtained from sample 20 DNA (Table 5) into BLAST yielded maximum match scores with DNA sequences from *Thekopsora rubiae*, *T. areolata*, *T. nipponica*, *Pucciniastrum epolobii*, *P. goeppertianum*. However, the highest amount of identity (99%) was found when matched against three *Thekopsora minima* sequences (GenBank Accession Nos. GU355675.1, [HQ661383.1](#), and [HM439777.1](#)). The query coverage was only around 44-48%, which resulted in the lower overall scores.

The low query coverage observed can be explained by the fact that the previously submitted *T. minima* samples were from DNA containing sections of 5.8S rRNA, the ITS2 region, and a section of 28S rRNA. As the sequence from sample #20 contained a larger region of DNA than these samples, consisting of the 18S rRNA ribosomal subunit and the ITS1 region in addition to these regions, this is not a surprising result and is in fact expected. Importantly, sample #20 also matched with 100% identity to leaf rust

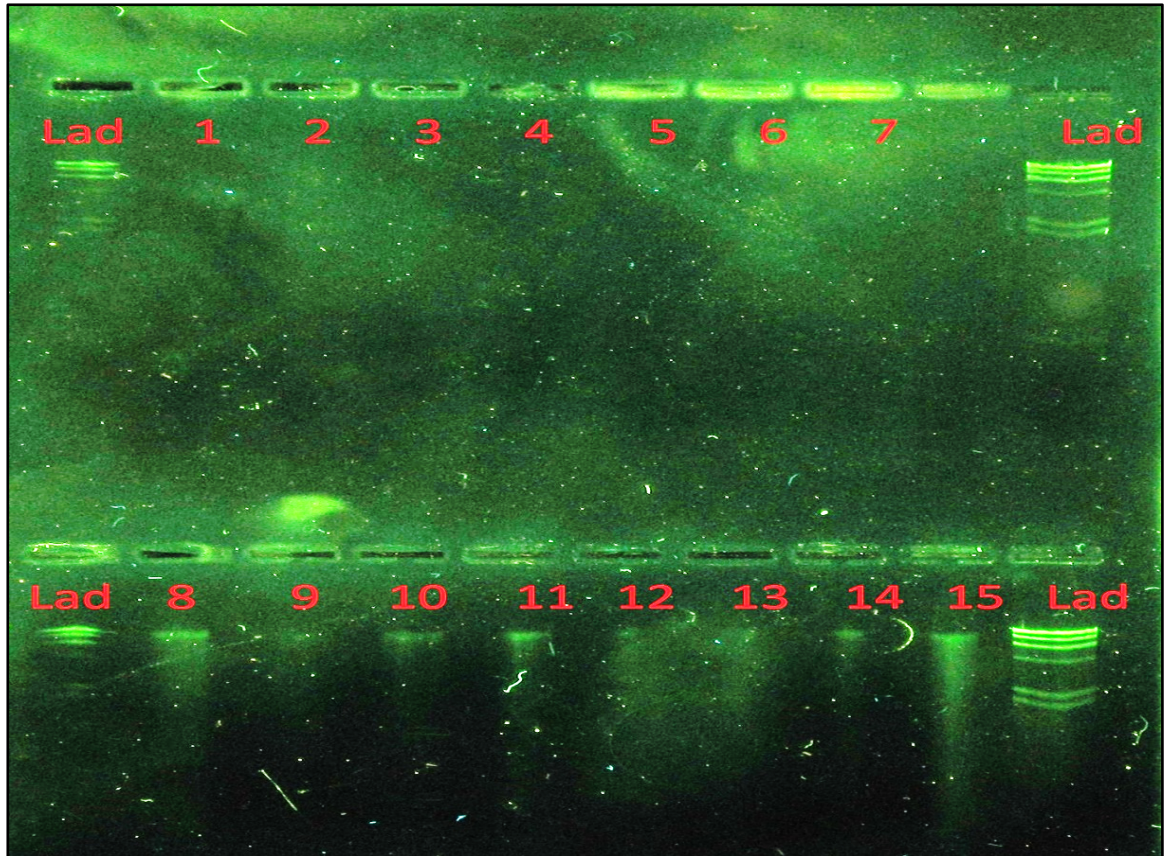
DNA isolated from the same field in 2014 (Horton, 2015), indicating the likelihood of this being the correct sequence for blueberry leaf rust. Taken together, this data indicated that the DNA from this particular sample is indeed from blueberry leaf rust.

The Clustal W application in BioEdit was used to align sample #20 (Table 5) with sequences that had the highest matching BLAST scores (Figure 7). Species represented by these high scoring sequences include *T. areolata*, *T. rubiae*, *T. nipponica*, *P. epilobii*, and *P. goeppertianum*. The ITS1 and ITS2 regions, as well as the 5.8S rRNA region were identified based on prior research (Hietala et al., 2014). For sample #20, the 5.8S rRNA gene starts around the 290<sup>th</sup> residue and ends near the 460<sup>th</sup> residue, and the nucleotide composition in this region was highly conserved. The 5.8S rRNA was flanked upstream by ITS1 and downstream by ITS2. The two ITS regions were replete with numerous point mutations and inserts. Thus, some of the nucleotide differences in these regions could possibly be used to distinguish *T. minima* from other rust species on the basis of their ITS sequences. The sequences for three variable sections of DNA are listed in Table 6 and could be used in the future to develop species-specific primers for *T. minima*.

Based on BLAST results against all sequences contained in the GenBank database, the two other sequenced samples (#16 and 17, Table 5, Figure 5) were concluded to be from the genus *Candida*, which is comprised of yeast species. When an alignment with samples 16 and 17 was attempted with sample 20 (confirmed to be *T. minima*), many gaps were needed to align the sequences, proving that they were not similar in DNA composition.

**Table 2.** DNA Quality from *T. minima* spore samples obtained with the Vacuum Method of spore collection. BBHF = Blueberry Hill Farm field site, WES = Wesley field site.

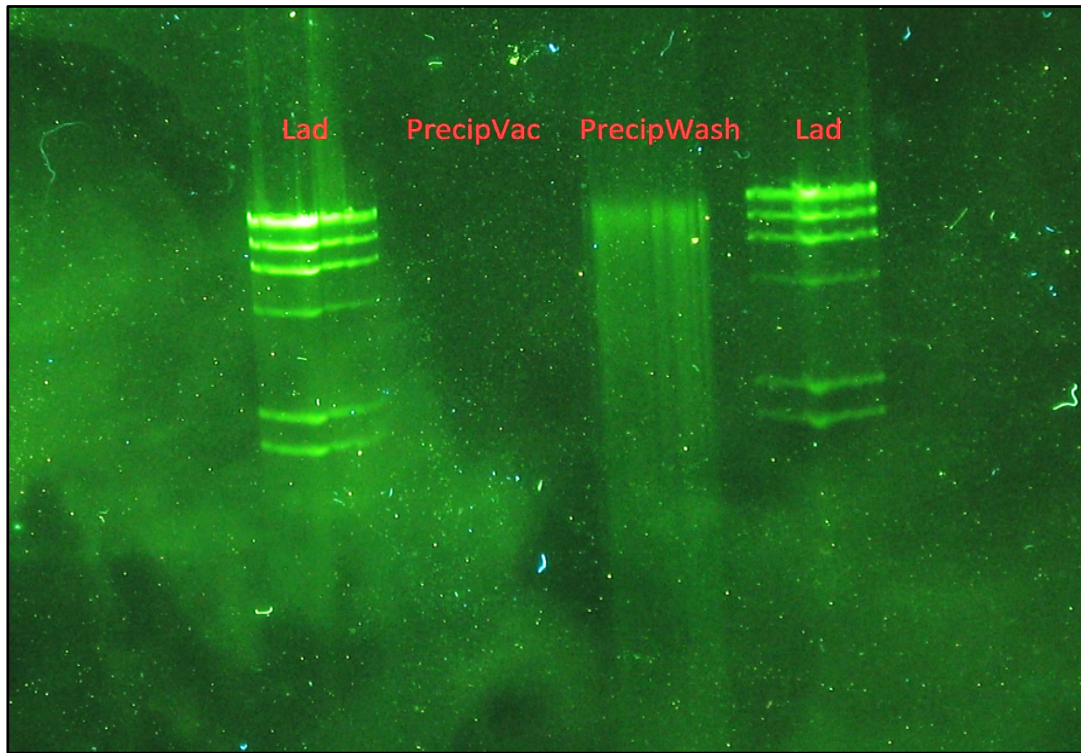
Code Name	Sample #	[DNA](ng/ $\mu$ L)	A <sub>260</sub> /A <sub>280</sub>	Band Visible	Extraction Procedure
BBHF2-9_9	1	5	1.47	No	Bead-Beating
BBHF2-9_10	2	7.5	1.04	No	Bead-Beating
WES-9_11	3	16	1.58	No	Bead-Beating
WES-9_14	4	6.9	1.74	No	Bead-Beating
WES-9_15	5	3.3	2.26	No	Bead-Beating
WES-9_16	6	2.7	1.62	No	Bead-Beating
WES-9_21	7	2.0	2.11	No	Bead-Beating



**Figure 3.** DNA Extracted from *T. minima* spores collected from Infected Leaves through Multiple Methods. Samples 1-7 collected through the vacuum method (Table 2) and samples 8-15 collected through the wash method (Table 3). Fields represented: BBHF (1-2), Wesley (3-7), East Machias (8-15). Lad is  $\lambda$  DNA/HindIII digestion for size reference.

**Table 3.** DNA Quality from *T. minima* spore samples obtained with the Wash Method of spore collection. EM = East Machias field site.

Code Name	Sample #	[DNA] (ng/ $\mu$ L)	A <sub>260</sub> /A <sub>280</sub>	Band Visible	Extraction Procedure
EM-10_16-1	8	25.1	1.49	Yes	Liquid N <sub>2</sub>
EM-10_16-2	9	18.1	1.47	No	Liquid N <sub>2</sub>
EM-10_16-3	10	24	1.49	Yes	Liquid N <sub>2</sub>
EM-10_16-4	11	26.1	1.47	Yes	Liquid N <sub>2</sub>
EM-10_16-5	12	23	1.44	No	Liquid N <sub>2</sub>
EM-10_16-6	13	30.8	1.44	No	Liquid N <sub>2</sub>
EM-10_16-7	14	37.9	1.44	Yes	Liquid N <sub>2</sub>
EM-10_16-8	15	31.2	1.46	Yes	Liquid N <sub>2</sub>



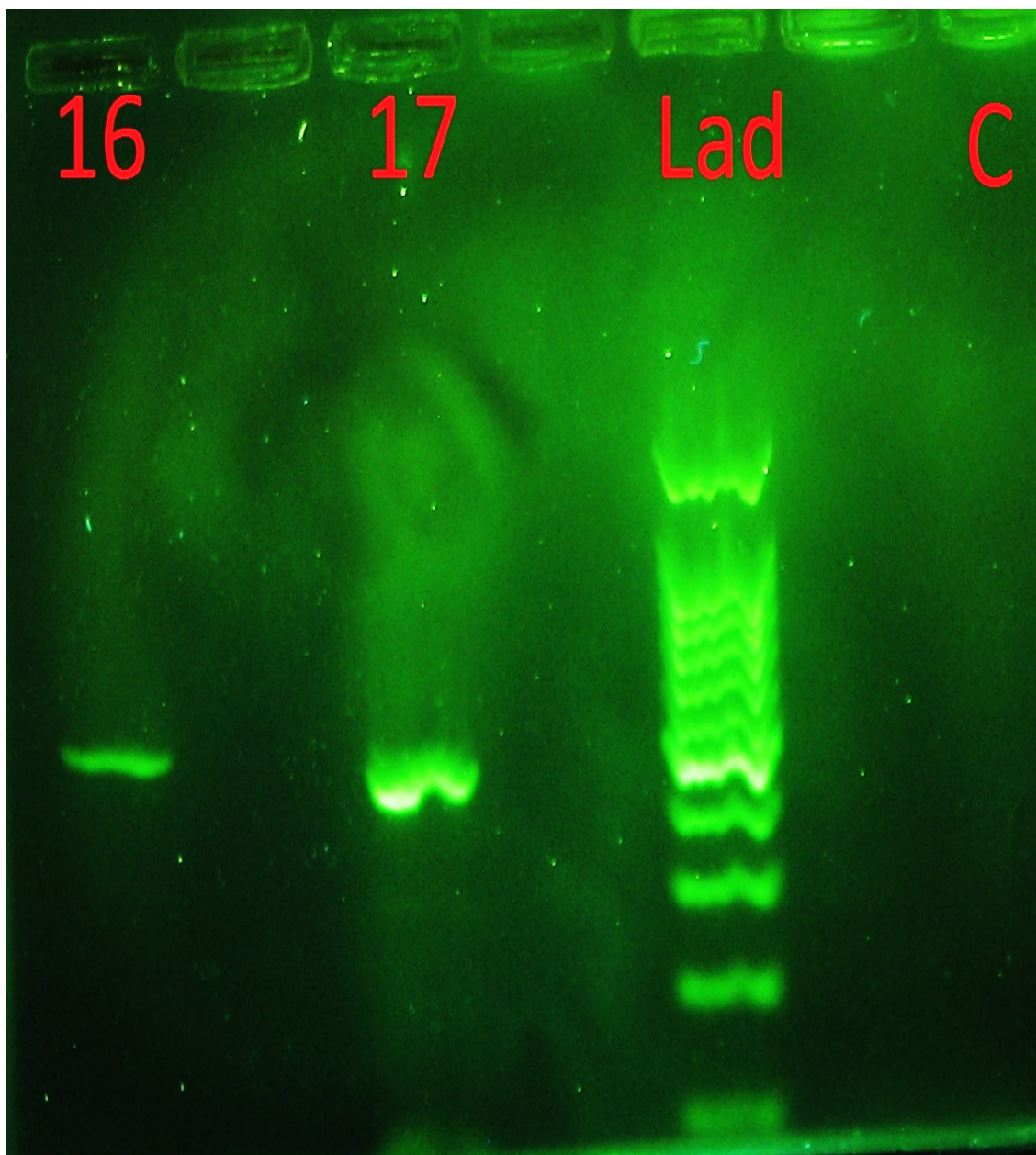
**Figure 4.** DNA concentrated from vacuum collected spores and washed leaf samples. PrecipVac is DNA from samples 1-7 precipitated together, PrecipWash is DNA from samples 8-15 precipitated together, and Lad is  $\lambda$  DNA/HindIII digestion for size reference.

**Table 4.** DNA Quality Data from Samples after Precipitation.

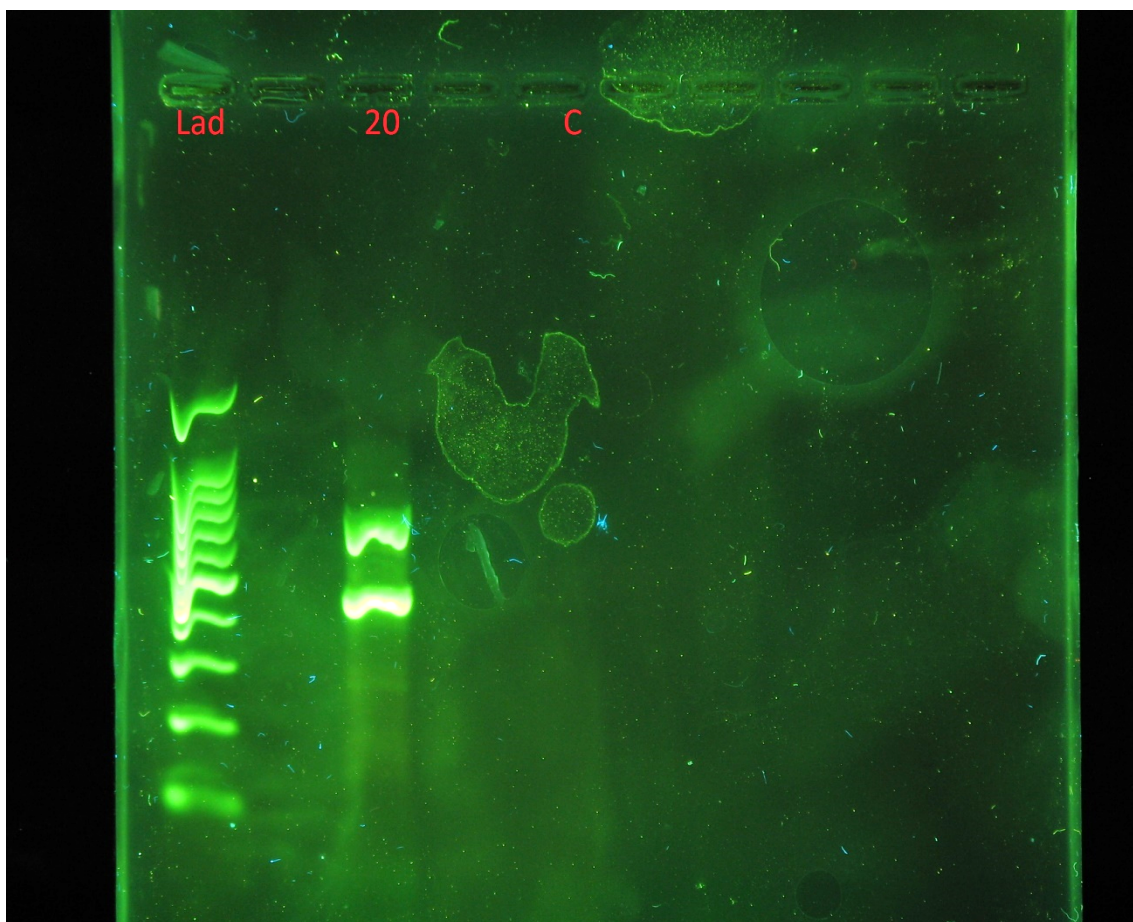
<b>Code Name</b>	<b>[DNA] (ng/μL)</b>	<b>A<sub>260</sub>/A<sub>280</sub></b>	<b>Band Visible</b>	<b>Extraction Procedure</b>
PrecipVac	3.7	1.16	No	Bead Beating
PrecipWash	137.5	1.45	Yes	Liquid N <sub>2</sub>

**Table 5.** DNA Quality from spore samples obtained with the Modified Vacuum Method and extracted by multiple methods. BBHF = Blueberry Hill Farm field site, WES = Wesley field site. LBBHF = Lower Blueberry Hill Farm field site.

<b>Code Name</b>	<b>Sample #</b>	<b>[DNA] (ng/μL)</b>	<b>A<sub>260</sub>/A<sub>280</sub></b>	<b>Band Visible</b>	<b>Extraction Procedure</b>
BBHF-11_16	16	4.4	2	No	Bead-Beating
BBHF-11_18	17	4.5	2.1	No	Bead-Beating
WES-11_19	18	6.3	1.85	No	Liquid N <sub>2</sub>
WES-11_20	19	8.2	1.55	No	Liquid N <sub>2</sub>
LBBHF	20	5	1.9	No	Bead-Beating and Cold/Heat Shock



**Figure 5.** PCR with DNA Extracted from Putative Leaf Rust Samples 16 and 17. ITS1 and ITS4 are the primers used in each PCR. Samples 16 and 17 are both from leaves from Blueberry Hill Farm, C is a negative control PCR with no additional DNA, and Lad is a 100 bp standard for size reference.



**Figure 6.** PCR with DNA Extracted from Putative Leaf Rust Sample 20. ITS1 and ITS4 are the primers used in each PCR. Sample 20 is DNA from spores sampled off leaves from Blueberry Hill Farm, C is a negative control PCR with no added DNA, and Lad is a 100 bp standard for size reference.

**Table 6.** Possible Sequences Unique to *T. minima* Compared to Other Rusts.

DNA Sequence	Nucleotide Residue	ITS Region
GAACCTCATTAAC	228-240	1
TATGGGATCTTGGAC	272-287	1
ACCATATAAGAG	569-580	2

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      10      20      30      40      50      60      70      80      90     100
LBBHF
DQ087231 T. areolata AAGTCGTAACAAGGTTCCGTAGGTGAACCTGCGGAAGGATCATTATTTAAATGTAAAG...CTT.AA..TT.
KC415802 T. rubiae -----TTTCCGTAGGTGAACCTGCGGAAGGATCATTATTTAAATTA---AAA.G.....C.....TAT.....A--.T.
KC415792 T. nipponica -----TTTCCGTAGGTGAACCTGCGGAAGGATCATTATTTAAACA---A--G.....A.....C.CTTAT.T..G...GAC.TC
DQ445906 P. epilobii AAGTCGTAACAAGGTTCCGTAGGTGAACCTGCGGAAGGATCATTATTTAGAAATC---AA.G.....TTT.A.CATA-.CT.
L76508 P. goeppertianum -----ATCATTTATTTAAAGTCA---AT-.....G..A..CTTT.AAT.-CTT.

      110     120     130     140     150     160     170     180     190     200
LBBHF
DQ087231 T. areolata AAACCAT-TATAAACCCAAAACCTGCTTGTGTGC-TCTAACGAGTATAGCATTTCAGTAGTACGCATCATGGACTTTATTGTTCAAGTTGCATTACCCCC
KC415802 T. rubiae ..T.A.CC-.T.....-T.....T.....G.....TC.T---T---GA.....
KC415792 T. nipponica .....-T.....G.....-T.....T.....G.....TC.....
DQ445906 P. epilobii .....CC.T.....G.....-T.....C.....G.....
L76508 P. goeppertianum ..C.....A.A.T.T.T.....AG.....

      210     220     230     240     250     260     270     280     290     300
LBBHF
DQ087231 T. areolata CC-TTATAAGTGACCCCTTTATTTTGAACCTCATTAACACTTAA---AAAGTTTAAAGATGTAAAT-TATGGGATGTGGAAC--TATATATAAC
KC415802 T. rubiae ..-C.T---TGTG.ATTGATGG...T.AAT..T.....C.AT..T---C.....
KC415792 T. nipponica ..-C.....T.....CGC...TCAT.-.-.....G.....AC.GAAAC...G.TATA.....
DQ445906 P. epilobii ..-C.....C.TA...TG.A.CTCA.T..C...CAAG.....CC---AAT.A..AT..A---
L76508 P. goeppertianum ..C.....ATCAT.A...GT..T.TGT.....C-C..T.GAA.-.-A---

      310     320     330     340     350     360     370     380     390     400
LBBHF
DQ087231 T. areolata TTTTAGCAATGGATCTCTTGGCTCTCAGATCGATGAAGAACAGTGAATGTGATAAGTAAATGTGAATTCAGAAATTCAGTGAATCATCGAATCTTTGA
KC415802 T. rubiae .....A.....T.....
KC415792 T. nipponica .....A.....G.....
DQ445906 P. epilobii .....A.....G.....
L76508 P. goeppertianum .....A.....

      410     420     430     440     450     460     470     480     490     500
LBBHF
DQ087231 T. areolata ACGCACCTTGACACCTTTTGGT-ATTCCAAAGGTACACCTGTTTGAGTGTGATGAACCCCTCTCATCATAATCTTTATTTA-----GT-TAAGAGT
KC415802 T. rubiae .....-.....TC.....G-----A.A.....
KC415792 T. nipponica .....-.....G..C.G.....-.....
DQ445906 P. epilobii .....C.....T.....C.G.....C-----T.....
L76508 P. goeppertianum .....C.....T.....TC.G.....A.....A.G..
.....-C.....TCC.G.CT..T...TATATATA.-A....T.

      510     520     530     540     550     560     570     580     590     600
LBBHF
DQ087231 T. areolata -TGGTGATGGATGTTGAGTGTGCGGTGCTGCTCACTTTAAATACATAAGTACTTTTATTGAGAAACCATATAAGAGTGGAGAAATACCTTGGTGTGA
KC415802 T. rubiae -.....C.....A.....T...TA-----A.A.....A.
KC415792 T. nipponica -.....C.C.....G.....C...AT----GA.A...T.TT.....
DQ445906 P. epilobii -.....A.....G.....G...CG..TGA-----A.A...T.T.....
L76508 P. goeppertianum C.....GTG.....T...-----TA.A.....

      610     620     630     640     650     660     670     680     690     700
LBBHF
DQ087231 T. areolata TATTTA-----TTATTCATCAAGGAGTGTGGTGGGGTTTACCTACTGCAGCCATT-GTT---TATTGATTTTTTGAT-----
KC415802 T. rubiae .....TGTT-----T.....A.TTAC--.A.....T...T----G...AAA.A.C.TCCTAACCCCTATTATATT--
KC415792 T. nipponica ...C-----GA...G.....A.G-----T...T...AAA.A.C.TCCTAACCCCGAGATATA--
DQ445906 P. epilobii .....ATTA--.G.A.....A.....A.T...TTGAC-CA.G...G.AAA.A.C.TCCTAACCCCATTTGAACCT
L76508 P. goeppertianum ...ATTATTATCA.A...TG.....A....A.CT--C-.....TT.GTGA.G...GAA.A.C.TCCTAACCCCATTTATAAT--
.....TG...A...AATA.C..GCTA.--.A.....T.AG---T...AAA.A.C.TCCTAACCCCAATTTCATT--

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**Figure 7. Alignment of Putative *T. minima* DNA against ITS Regions of Other Rust Samples.** DNA from LBBHF sample 20 is shown against sequences from *T. areolata* (DQ087231), *T. rubiae* (KC415802), *T. nipponica* (KC415792), *P. epilobii* (DQ445906), *P. goeppertianum* (L76508). The line symbol (–) indicates a gap in alignment, and a dot (.) denotes identical nucleotides between sequences at any given position. GenBank ascension # is given in parentheses.

## Discussion

Many alterations that needed to be made in the methodology as this research progressed. Obtaining leaf rust DNA, as well as the spores themselves, was particularly difficult and many modifications needed to be made in order to find an effective method to accomplish this task. This is likely due to the fact that many organisms other than *T. minima* exist on the leaf, and it can be difficult to target rust spores alone for harvesting. Additionally, there is the problem of the thick cell walls of the spores, which makes it hard to extract DNA from them. The modified vacuum method, along with a “cold/heat shock” of the spores before bead-beating them, appears to result in DNA of high quality from the spores. This may be due to a more effective weakening of the cell wall, but the exact cause is unknown. Overall though, collection of spores was time-consuming and thus did not meet the objective of developing a method for the rapid detection of *T. minima* in blueberry leaf samples. It would not likely provide the high amount of throughput needed to develop such a method, but did allow for the comparison of collected DNA sequences with previously submitted sequence data. These data reveal regions of DNA unique to *T. minima* when compared to other rusts and confirm the presence of this species of rust fungus in blueberry fields of Maine.

The results exemplify the difficulty in isolating leaf rust spores from the leaves of lowbush blueberry, but they also reveal insights into optimal methods for both the collection of spores and the extraction of DNA for further experimentation. Spore samples collected with the first method, the vacuum method, had DNA low in overall concentration and quality. Explanations for this include the vacuum pressure being too low, as well as experimenter not harvesting all possible leaf rust spores from the leaves

during the process. Additionally, spores may have also been lost at the end of procedure during the final collection of material with Tween 20. Given this information, it would not be recommended to use this method in the future.

On the other hand, it was shown that the wash collection method, while yielding lots of DNA, may not be ideal for collecting leaf rust spores only. The precise composition of total material collected through this method was unknown and of concern, as there was no strict measure employed to ensure that only rust spores were washed into the collection vessel. The samples had relatively high DNA concentrations after extraction, but low  $A_{260}/A_{280}$  ratios, indicative of impurity. Improper removal of proteins and other macromolecules may have decreased the purity and altered absorbance readings. Fungal DNA obtained this way could not be amplified consistently with the universal fungal ITS primers ITS1 and ITS4. Possible reasons for this difficulty include an incomplete removal of PCR inhibitors, a non-ideal amount of total DNA in the reaction mixture, and contaminant DNA from nonfungal species. In conclusion, the wash method as described here would not be an ideal way to specifically obtain *T. minima* DNA from blueberry leaves that is suitable for PCR.

A superior method might comprise of a slight modification of the vacuum line, which involves cutting the pipette tip before vacuuming. It is believed that this allowed for a decreased flow velocity from the vacuum, and the spores obtained this way generated DNA that was able to be amplified by the universal primers. This could be due to the fact that more spores were collected overall, as less were blown away from the pipette tip. With this in mind however, it may still be helpful to pool multiple sources from the same field into a larger sample to provide a greater quantity of spores for DNA

extraction. This is because initial DNA yields were inadequate when small amounts of leaves were sampled. Only after combining multiple spore samples from a field was an adequate DNA amount and quality obtained. Additionally, an optimal method for DNA extraction may also include an added freezing/heating step before pulverization with the beads. The sequence data in this research was derived from DNA obtained in this way. It is believed that this “cold/heat shock” procedure may aid in the disruption of the spore cell wall for proper diffusion of DNA into extraction buffer. It was successful in obtaining quality DNA for this study and is recommended as a starting point for future experiments.

DNA sequence data from samples 16, 17, and 20, amplified using PCR, shows the problem of contamination that arises trying to detect this particular pathogen. In particular, the genus *Candida* may represent a large source of contamination based on the findings of this study. As these yeasts are ubiquitous in air samples, and a species of *Candida* was even found to grow specifically on blueberry leaves (Tokuoka et al 1987), this result should not be surprising, but gives insight into the complicated nature of the fungal ecosystem on the leaves of a lowbush blueberry plant. In addition, these yeasts may not be on each individual blueberry leaf of a sample, but will be high enough in quantity to contaminate the majority of a field (Tournas and Katsoudas 2005). There must be ways developed to circumvent this issue. One way to achieve this is to take samples from natural sources so that a method can specifically target fungal spores on the surface of leaves, without collecting other contaminants. This could be attempted through certain methods delineated here, but would not guarantee the collection of *T. minima* spores alone.

The other conclusion that can be made from the DNA sequence data is that sample 20 was *T. minima* and serves as confirmation of a previous discovery of this species of fungus at Blueberry Hill Farm (Horton, 2015). Indeed, the nucleotide composition of the ITS regions of this isolated sample was identical to that discovered last year in the same field. The ITS regions of sample 20 are seen to be variable from a select group of other rust species (Figure 6 and Figure 7), and presumably from other fungal species that may reside on the blueberry leaf. These DNA sequence differences could possibly be used to design primers that would have specificity for *T. minima*.

While the sequences listed in Table 6 are likely too short to make an ideal primer (Apte and Daniel, 2009), they may provide a starting point for future research. Ideally, they would be longer than what is shown in Table 6 (around 18-30 bp) and compared to sequences from more species. As with any primer development process though, consideration will also need to be given to length, melting temperature, GC% composition, nucleotide repetition, and inter/intra-primer homology. Furthermore, it is important to ensure the ends of a primer, and the 3' end in particular, are composed of unique bases as terminal mismatches can drastically reduce yield (Yang et al., 2006). Certain bioinformatics programs have been developed to test these predictions and could be utilized for this task. After this has been researched thoroughly, specificity can then be determined by testing the ability of a primer pair to discriminate against other fungi common to downeast Maine.

In this study, when the confirmed *T. minima* sequence (sample 20) was compared to *P. goeppertianum* DNA, differences in nucleotide sequence were seen. This is a critical observation because Witch's Broom, the disease caused by *P. goeppertianum*,

also damages lowbush blueberry plants (Caruso and Ramsdell 1995). Interestingly, it has also been suggested that the *Thekopsora* genus is polyphyletic (Maier et al. 2003). As this would suggest that other rust genera are closely related to *T. minima*, the DNA sequences of other rust species may be more similar to *T. minima* in nucleotide sequence than previously believed. Thus it will be important to ensure that minute differences in sequence are exploited in the future such that any molecular diagnostic test employed is specific for *T. minima* alone and detects this particular pathogen only.

The primer pair deployed in this study (ITS1/ITS4) is not going to be able to differentiate between rust species. One could therefore consider the possibility of pairing a newly designed primer with one of these so-called “universal primers.” This would allow researchers to exploit regions unique to *T. minima* while ensuring the proper binding of the forward or reverse primer. For example, ITS4-B was designed to amplify DNA from *Basidiomycota* species when used with ITS1 (Gardes and Bruns, 1993). It was successful in amplifying the fungal DNA of a rust species from rust-infected plants, even when plant DNA was also present. The ITS region thus remains a good region to continue the hunt for a dependable primer for the blueberry leaf rust fungus. The variable ITS regions, surrounded by conserved rRNA sequences, can be used to find regions that are ideal for primer binding, and the conserved sequences will be known to reside in all fungal species (White et al., 1990).

Once a primer pair has been shown to accurately and specifically target *T. minima* DNA, quantification of spores could be carried out with qPCR as in other studies (Hietala et al., 2014, Fountaine et al. 2010). This could serve as an assessment for the severity of disease within a given field, and also help create a plan for when to test for dispersal of

leaf rust. This could be done by examining correlations between rust spore amounts and weather data (i.e. temperature, humidity, moisture, wind, etc.) for a particular location. Upon making these sorts of inferences, detection of spores should become quicker and easier. As of now, infection is usually not inferred until mid-July and subsequent leaf damage lowers potential yield. If spore detection was able to indicate infection before this time, procedures could then be carried out to prevent disease progression.

The ability to track spores, and couple that data with weather information, should allow for an increase in the accurate detection of leaf rust spores. Accurate detection would allow for disease inference sooner than infection currently becomes apparent. This would be greatly beneficial to blueberry growers as there are increasing concerns about the negative consequences of fungicides and other chemical control measures. More effective targeting with fungicide applications would improve crop yield and lower the costs associated with these external control measures. A molecular method coupled with weather data could potentially reveal key elements of the rust life cycle, helping the farmers of Maine increase the effectiveness of their fungicide applications. It is my belief that primers designed based on the results presented here will help them achieve that goal.

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## Author's Biography

Wells, Maine is where Steven Valentino spent most of his childhood, alongside his parents and brother Peter. His undergraduate collegiate experience began in 2012 when he enrolled as a Molecular and Cellular Biology major at the University of Maine. Later, he added a minor in mathematics to supplement this course of study. His hobbies have remained incredibly consistent over time over the years, which include reading, writing and photography/film. In 2014, he submitted his novella *Life in a Trashcan* to the Amazon and iBooks marketplaces and added another, *Poems by Valentino*, in 2015. Another thing that Steven values are his family and friends, including his fiancée Elizabeth whom he will wed in July 2016. Having a wide range of interests, he is currently indecisive as to a definite career path, but has strongly considered pursuing a degree related to biostatistics in the near future. In the meantime, he plans to continue working in the biological sciences.