PLANT DISEASE DIAGNOSIS IN THE AGE OF PCR: IMPROVING THE TOOLBOX AND INCREASING CREDIBILITY

By

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To Phil, with gratitude for this big full life and for team Us; and to Emma, whose grin kept me striving for the finish line.

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LIST OF ABBREVIATIONS

APHIS	Animal and Plant Health Inspection Service
APS	American Phytopathological Society
CPDN	Caribbean Plant Disease Network
CSREES	Cooperative State Research, Extension, and Education System
DNA	Deoxyribonucleic Acid
IPDN	International Plant Diagnostic Network
NIFA	National Institute of Food and Agriculture
NPDN	National Plant Diagnostic Network
PCR	Polymerase Chain Reaction
PDC	Plant Diagnostic Center
SOP	Standard Operating Procedure
SPDN	Southern Plant Diagnostic Network
STARD	System for True, Accurate and Reliable Diagnostics
USDA	United States Department of Agriculture

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Plant disease diagnosis necessitates the application of methods to identify the causal agent of disease in a plant sample. The diagnosis itself is a culmination of processes including technical assays, review of relevant literature, critical thinking and scientific curiosity, and logical assessment of hypotheses. Polymerase Chain Reaction (PCR) is increasingly utilized as a powerful identification tool, but without standards and appropriate controls, the results can be misused and misinterpreted and their relevance to a diagnosis decreases. Barriers to widespread adoption of PCR identification in many diagnostic laboratories include nucleic acid extraction and amplification inhibitors, difficulty in obtaining positive controls, and lack of data regarding the reliability and accuracy of the protocol. In two rounds of testing at five land-grant university plant diagnostic laboratories, two commercially-available nucleic acid extraction kits were found to remove most amplification inhibitors, including humic acid. Spectrophotometric analysis of crude kit-based DNA extracts indicated that protein contamination and DNA concentration were not correlated to amplification, but DNA free of phenolic contamination was correlated to amplification. Cloned DNA positive controls were developed and tested. Analysis of the results of these tests was incorporated into the

development of a model to determine the appropriate number of true positive and true negative samples that a lab must process to validly test a protocol. Lastly, the work herein contributed to the refinement of five guidelines for development of methods for diagnostic applications. Our results indicate that PCR can be a useful identification tool in the diagnostic setting when appropriate controls and validation are included in the development of the method. Additionally, it is possible to easily determine the minimum number of blind-panel samples required for a valid test, based on the risk associated with false positives and false negatives. Our research has addressed some barriers to incorporation of PCR into the diagnostic toolbox, which may encourage further discussion of molecular method development and adoption in plant diagnostic laboratories.

CHAPTER 1
THE PLANT DIAGNOSTIC LABORATORY: THE CORE OF PHYTOPATHOLOGY

Introduction: Phytopathology and Disease Diagnosis

Plant disease diagnosis requires a bit of history and a long perspective on pathogens, plant diseases, and the formation and support of extension and diagnostics. A medical practitioner by education, Anton DeBary applied his training in diagnosis to the field, literally; he followed the progress of an organism (*Phytophthora infestans*) from spore to spore on potato leaves, stems, and tubers as the symptoms of disease appeared and progressed. Erwin F. Smith, in the first article of the first volume of Phytopathology, describes DeBary as instrumental to the formulation of phytopathology, "Of all the personalities contributing to the advancement of plant pathology from its crude beginnings [1861] to the present time [February 1911], none has been more interesting than that of De Bary, none more productive of important results." (Smith E., 1911) Indeed, the classic plant pathogen that caused the Irish potato famine and is credited as the beginning of plant pathology bears his name, *Phytophthora infestans* (Mont.) deBary, forever tying the introduction of phytopathology to its founder. Smith himself was instrumental to the development of phytobacteriology, and published on crown gall in that seminal volume, describing experimental evidence that the disease was due to bacterial infection, and not to fungi, mites, environment, or nutrition. In short, both of these phytopathological founding fathers were quintessential diagnosticians, using the tests at their disposal, as well as their own experience and scientific curiosity to discover the causal agents of disease.

Like many of his contemporaries of the early 20th century, Smith's findings on plant infections were first published in other, general journals such as *Science*, or

discussed in presentations to societies dedicated to human medicine, such as the American Society for Cancer Research. The American Phytopathological Society (APS), at the time of the primary publication of its premier journal, was just three years old, but phytopathological publications were already numerous in the US and around the world. By this time, fifty years of study had occurred to define phytopathology, and the very definition of a plant pathogen continues to be discussed to this day.

Although Koch's postulates (Koch, 1890) as translated in Relman (1996) may still be used to prove pathogenicity of culturable organisms, the postulates do not in themselves define what a pathogen is. Koch's goal in presenting his guidelines was at least in part to define a standard thought process that would prove as far as biologically possible that a specific microbial parasite was causing the disease. B. M. Duggar noted that in the early 1900s most scientists would concede that disease is recognized as abnormal cellular activity in response to some sort of stimulus (Duggar, 1911). However, this describes only the plant's side of a complex equation and does not necessarily involve pathogens.

For the purposes of this dissertation, we will focus on those organisms which cause detrimental effects on the plant, and make the distinction between abiotic disorders and biotic diseases. Although one could argue that a virus might not qualify as an organism, there is little debate regarding its ability to cause plant disease. Furthermore, we will wade into the gray area of plant-parasite interactions and include nematodes but not arthropods in our discussions, with the full knowledge that the very definition of a pathogen is somewhat in the eye of the plant-holder.

APS includes nematodes in many of its materials including those in the APS Education Center resources, and Lambert and Bekal (2002) note that most plant-parasitic nematodes are “soilborne root pathogens”. Malcolm Shurtleff defined inoculum as “a pathogen or its parts (e.g. fungal spores or mycelium, nematodes, or virus particles, etc.)…” (Shurtleff, Glossary of plant-pathological terms, 1997). It should be noted that Nathan A. Cobb, the “father of nematology” in the US, was a plant pathologist (Huettel, 1991). The fact that nematodes molt and are related to insects at the kingdom level blurs the line between pathogen and parasite, in much the same way that the oomycetes are not truly fungi, but are more closely related to algae.

We acknowledge that there continues to be debate regarding abiotic disorder or injury versus disease in that injury is distinguished from disease in timing; injury, often from an abiotic/mechanical or arthropod source, is limited to a short duration (cuts made by farming implements, chewing injury from caterpillars, etc.), whereas disease progresses over time. However, some abiotic conditions may produce symptoms that develop over time (continuing micronutrient deficiency, heat-stress that induces necrotic leaf spots and increases in magnitude through the summer season) and some diseases may be caused by pathogens that infect but, due to unfavorable environmental or physiological conditions, produce symptoms that do not progress.

In drawing the line in the sand marking disease as caused by a pathogen with infection and symptoms that progress over time, we respectfully disagree with the conclusions derived by Wallace (Wallace, 1978) and Grogan (Grogan, 1981) that includes abiotic injuries and disorders. However, we fully support the idea that a diagnostician must include abiotic disorders and other injuries (insect or mammalian

feeding, etc.) in the holistic diagnosis and management of the plant problem. Our focus will be, for the most part, on plant pathology as it relates to service to society through the detection, diagnosis, and eventually management of the causal agents of plant diseases.

Detection, Diagnosis, and Scientific Service

Detection and diagnosis are two different concepts. The Merriam-Webster dictionary defines detection “to discover or determine the existence, presence, or fact of” something. The word is derived from *detectus* (Latin), “to uncover”. Diagnosis, on the other hand, is defined as “the art or act of identifying a disease from its signs and symptoms” and “the investigation or analysis of the cause or nature of a condition, situation, or problem”. The Greek origins of the word diagnosis indicate a meaning of knowing or distinguishing. In practical terms, organisms are identified and detected, whereas a disease is diagnosed following a synthesis of information pertaining to the pathogen, host, and environmental parameters of a given situation.

The service provided by the practice of applied plant pathology is ultimately the management or abatement of a disease, thus detection and diagnosis are simply part of a larger process, as noted by Barnes (Barnes, 1994) and others. Diagnosing a plant disease requires recognition of abnormal. An effective diagnostician takes into account horticulture and botany to identify “normal” for a particular plant and management.

The process of diagnosis is often noted to include science and art (Grogan, 1981); appropriate training and techniques coupled with experience, critical thinking skills, and scientific curiosity shape the diagnosis.

Ruhl (Ruhl, 1982) noted that the widespread existence of what we consider a plant diagnostic clinic in the US is relatively recent, dating for the most part to the

1960s, although a select few were instituted in the late-1800s and early-1900s. Barnes' review (Barnes, 1994) of the plant diagnostic clinic's role in education and service indicates that although today's diagnostic clinics are often formal programs unto themselves, the clinics also serve as a point of intersection of plant health specialists, diagnosticians, and their clientele, as well as a place of plant health program integration.

The mission of those diagnostic clinics associated with the Cooperative Extension Service continues to be service to each state's citizens as an outreach mechanism of the land-grant mission. Signed into law in 1862 by President Abraham Lincoln, the Morrill Act (http://www.csrees.usda.gov/about/offices/legis/legis_statutes.html) donated public lands to each state, which in turn sold the land and used the funds to build public universities focused on agricultural and mechanical arts. A second Morrill Act approved by Congress in 1890 provided continuing funds for agricultural and mechanical education (amended in 1981 to "food and agricultural sciences") at the previously-established "land-grant colleges". The Cooperative Extension Service was formalized in 1914 via the Smith-Lever Act (Smith L., 1914), which established and funded the cooperative relationship between the federal agricultural agency, USDA, and the land-grant colleges to develop practical applications of research outputs and to instruct the public via field trials and other practical demonstrations.

Today's extension plant disease clinics are an outreach mechanism for implementation of research-based diagnostic and detection methods and dissemination of locally field-tested management recommendations. These clinics often operate in tandem with state departments of agriculture, whose mission relates to detection,

diagnosis, exclusion, quarantine, and eradication of regulated organisms. Although Ruhl noted that most of the clinics originally were set up to assist extension agents, direct service to clientele has rapidly surpassed the samples from agents, likely due in some part to the visibility and capacity provided through programs such as the National Plant Diagnostic Network (NPDN).

The National Plant Diagnostic Network

Established as a program within the USDA Cooperative States Research, Education, and Extension Service (CSREES) by the US Secretary of Agriculture in 2002, the NPDN (then called the Animal and Plant Disease and Pest Surveillance and Detection Network) established formal linkages between state diagnostic services (mostly at land-grant universities) and a regional hub for five regions of the US (Stack et al., 2006). These networked laboratories, representing all US states and territories, were given objectives including early detection and accurate diagnosis of high-consequence disease and pest outbreaks via education of a cadre of first detector, training and capacity-building in the diagnostic laboratories, and reporting of diagnostic data to a national repository.

The hub-and-spoke system provides coordination of training and reporting, as well as surge capacity during time of high sample submission (Cardwell, 2009). At the local level, the diagnostic clinics interact with their traditional counterparts in extension and agriculture. At the national level, the NPDN system is improving capacity and communications and building upon the existing Cooperative Extension Service.

Sharing of protocols, ideas, and reports of new pathogens has increased the specificity of diagnoses and reduced the turnaround time for high-impact pathogens (Pers. comm. members of APS Diagnostics Subject Matter Committee, APS national

meeting, 2010). The NPDN program funds have been leveraged to support purchases of new equipment and supplies, and hiring of part-time diagnostic staff (Stack, et al., 2006). As a result, the clinics have increased capacity in diagnosis through traditional and molecular methods (Honeycutt, 2011).

Training the Next Generation of Diagnosticians and Applied Plant Pathologists

"The advance of plant pathology will be just in proportion to the clear recognition that its chief problems are biological rather than economic… This means grappling with the problems of the most varied nature and exceeding complexity. And the variety and complexity promise to increase with the progress of the work. Success along these higher lines is at present less than it should be because of lack of specially trained investigators. In years to come the need for such training will become still more imperative." (Jones, 1911). It is interesting that L.R. Jones wrote this in 1911; it could be said of 2011 as well, especially in relation to applied phytopathology and plant disease diagnosis. His discussion in Phytopathology lobbied for broad scientific training at both the undergraduate and graduate level, to include basic physical and biological sciences, and of course focusing on phytopathology and emphasizing research such that the graduate can see the forest and the trees, so to speak.

The outcome should be that one will contribute to science as a whole and our understanding of our discipline as a science and a service. Faculty with crop- or organism-specific research and extension responsibilities often serve as subject-matter experts to the diagnostic clinics, increasing the accuracy of diagnoses and the utility of management recommendations. However, these experts have been decreasing in number due to retirements and reassignments (Everts, 2012), new faculty have different expectations put upon them by their academic administrators and extension clientele

(Stack, et al., 2006), and some academic departments are geographically distributed such only digital consultation is possible.

Doctoral and post-doctoral research is increasingly focused on basic research; training in pathogen systematics and field identification is declining. To increase their students' exposure to applied plant pathology, some faculty are encouraging interaction with the plant disease clinics. Most of the diagnostic clinics in the Southern Plant Diagnostic Network (SPDN) employ and train graduate students (SPDN survey, July 2012) as part of their staff and in support of the educational mission of their academic departments. At least two have formal for-credit internship programs in plant disease diagnosis (LSU AgCenter and UF).

Barnes (Barnes, 1994) discussed the role of teaching and training in his review of North American diagnostic clinics. Directors and staff of the diagnostic clinics reported it was with some difficulty that they were balancing the extension needs of diagnostic clinic clientele, which require timely diagnosis and communication on many samples each day, with the educational needs of graduate students, who require long periods of one-on-one training in utilization of methods, equipment, and other lab resources, as well as instruction in critical thinking and the process of diagnosis. Both the APS Diagnostics Subject Matter Committee and the NPDN are working on the issue of training the next generation of diagnosticians, and indeed the topic of training the next generation of plant pathologists is such an important one that the APS has developed an online journal (*The Plant Health Instructor*) and accompanying online resources (APS*net* Education Center) dedicated to the education of future plant pathologists.

Detection of Plant Pathogens

Detection of plant pathogens is becoming both increasingly important and challenging as global commerce and climate change converge, resulting in the distribution of new hosts and their accompanying pathogens. Early detection and accurate diagnosis encourages appropriate management recommendations and actions ranging from eradication to the use of targeted fungicide spray programs.

Standardization and Speed

We have an immediate need to quickly and accurately detect high-impact pathogens, but streamlined diagnostic protocols do not exist for most of the more recalcitrant and emerging pathogens. The development of the National Plant Diagnostic Network (NPDN) and other diagnostic networks (IPDN, CPDN) has increased capacity for plant disease diagnostics worldwide (Miller, Beed, & Harmon, 2009). However, the continuing need for rapid, standardized, and efficient diagnostic tools and methods is clearly evident in the request from NPDN diagnosticians for DNA extraction and serological kits, streamlined PCR protocols, and high-throughput laboratory methods (National Plant Diagnostic Network Diagnostician survey, NPDN Diagnostics Committee, 2009, unpublished). Surveys of the thirteen member state diagnosticians of the Southern Plant Diagnostic Network (SPDN, November 2008 and March 2009, (table 1-1) indicated diagnosticians would be likely to use PCR if the following conditions could be met 1) published protocols are evaluated, adapted for diagnostic laboratory use, and gathered into a single easily-accessible online source, 2) PCR-positive controls are accessible, 3) training is provided to refresh PCR skills and disseminate protocols and troubleshooting tips, and 4) an easier, less expensive and comparable kit or protocol

does not already exist (immunostrip kit, easily culturable and microscopically identifiable pathogen, etc.).

Use of Standard Operating Procedures (SOP) is one means to standardize diagnostic methods used and encourage trust in diagnostic results (Miller, Beed, & Harmon, 2009). NPDN personnel, in cooperation with subject matter experts and regulatory partners, developed several for diagnostic laboratories. Prioritization of SOP development has focused on high-impact and select agent pathogens. The current SOPs can be found on the NPDN website (www.npdn.org).

Many available protocols are based on research laboratory methods and do not take into account the need for "off-the-shelf" streamlining to accommodate the plant diagnostic laboratory. Most of the NPDN laboratories have conventional PCR, but only a select few have real-time PCR due to the initial expense and annual maintenance costs of the required equipment and consumables. Additionally, diagnostic clinics receive a huge variety of individual samples, whereas research laboratories process groups of the same type of sample. This indicates a need for diagnostic PCR protocols that are both robust and streamlined so the diagnostician does not have to relearn the intricacies of many different protocols each time a sample comes into the laboratory. The use of DNA extraction kits, standardized protocols, and bead or lyophilized formulations that include all components except primers, target DNA, and water, will encourage standardized diagnostics. Leveraging of existing equipment and knowledge will increase capacity of NPDN and other diagnostic laboratories and reduce the costs per sample to a reasonable amount for diagnostic laboratory clientele. Sharing the protocols with international partners will increase diagnostic capacity for exporting

countries, reducing the risk of importing those diseases into the US. Standardized protocols and quality internal controls will increase sample processing capacity of all laboratories, not just within the NPDN and will be easily incorporated into the future accreditation program of the NPDN. Involvement of diagnosticians in the development and testing of such assays should increase adoption of standardized diagnostics. This will enable U.S. plant diagnostic facilities to better detect import, export, endemic, and emerging pathogens as needed.

Molecular Methods: Improving Identification of Difficult Pathogens

NPDN diagnostic laboratories are positioned to be front-line for the recognition of new diseases and pathogens since diagnostic laboratories are the growers' mainstay for the rapid and accurate identification of causal agents for new and known diseases. While some pathogen groups are addressed, diagnostic protocols for obligate, recalcitrant, and emerging pathogens are inadequate. The application of molecular identification tools can help to address this deficiency.

The acute lack in U.S. diagnostic laboratories of appropriate assays for new and emerging viruses is worrisome (J. Polston, pers. comm.). Immuno-based assays such as ELISA and Immunostrips™ are generally narrow and limited to the detection of a single genus or sometimes very closely related species. Additionally, these assays are designed to detect known pathogens, not to identify new ones. Microarrays, which have been proposed as a solution to pathogen diagnostics, are extremely expensive and the chips are not available yet for most pathogens; furthermore, their sensitivity with environmental samples such as extracts from diseased plant tissue is limited.

Fungi are often identified simply by culturing on general or semi-selective media followed by microscopic observation. Some fungi, however, are not amenable to this

method, as they are either obligate parasites and cannot be cultured away from the host, or do not sporulate under normal diagnostic laboratory conditions, and thus an important diagnostic clue is eliminated.

Many bacteria require multiple selective media or conditions to elucidate their genus and species (Bergey, 1994) (Schaad, Jones, & Chun, 2001), and new bacterial diseases require further molecular or serological identification and testing on host material. Additionally, fastidious bacteria are cultured with difficulty (*Xylella* spp.), or at present cannot be cultured at all ('*Ca*. Liberibacter spp.').

Nematode diagnosis has traditionally relied on time-consuming extraction and measurement of the population present in a set of soil or plant samples. As with diagnosis of other disease-causing organisms, identification is complicated by the presence of multiple species or genera in the same samples.

All of the above diagnostic methods rely on the existence and ideally local availability of reference specimens to act as positive controls and comparative resources. Laboratory identification and reference manuals abound for plant pathogenic bacteria (Bergey, 1994) (Schaad, Jones, & Chun, 2001), fungi (Barnett & Hunter, 1998) (Leslie & Summerell, 2006) (Dugan, 2006) and nematodes (Mai, 1996) (Taylor & Sasser, 1978) (Luc, Sikora, & Bridge, 2005), that explain the utility and interpretation of the above methods and are in heavy rotation in the majority of diagnostic clinics.

Identification to genus is generally adequate for the majority of diagnoses, since the end-result management recommendation may be the same for nearly all species within a genus. Identification to the species level requires time and expertise that may

not be available in a diagnostic clinic; however, situations that would benefit from this level of classification include the diagnosis of new strains or species, and any regulatory identification, which must have species-level identification. In these cases, the diagnostician may need to refer the sample to an expert-level identifier, who may not have time or even exist, or turn to other methods for identification, such as molecular detection, if available. The need to culture, count, measure, or otherwise "see" a disease-causing organism has not been reduced by the introduction of serological or molecular tests; rather, these tests complement the traditional ones, and add to the confidence of a diagnosis.

One More Tool for the Diagnostic Toolbox: PCR as Part of Multi-test Diagnosis

The Polymerase Chain Reaction (PCR) (Mullis, Ferre, & Gibbs, 1994) has enabled scientists to detect the presence of minute amounts of organism deoxyribose nucleic acids (DNA). During his lecture (Mullis & Smith, 1993) at the acceptance of the Nobel Prize in chemistry, Mullis notes that the world of molecular investigation was founded on the publication (Watson & Crick, 1953) of the Nobel-prize winning work defining the molecular structure of DNA. This technology has immense potential as an identification tool in the plant diagnostic laboratory (Martin, 2000) (Miller S. A., 1988) (Powers, 2004).

The Polymerase Chain Reaction (PCR)

PCR is the process that occurs when DNA polymerase synthesizes new DNA strands that complement existing template DNA (Walker, 2010) (Michael Innis, 1990). The polymerase recognizes the 3' hydroxyl group of a short piece of DNA called a primer, to which it then adds additional complementary deoxynucleotide triphosphate molecules (dNTPs) to create double-stranded DNA with the single strand of the

template DNA. The same process occurs to the complementary strand of the double-stranded target DNA, delivering two new double strands for each round of PCR, each starting near the 5' end of the target strand, at the point of the 3' end of the primer.

The reaction occurs at three different temperatures, each designed to accommodate the activity of the polymerase, the specific bases within the primer sequences, and the length of the desired amplicon. This set of temperatures is cycled multiple times, exponentially increasing the target. The first temperature is 90ºC or slightly higher, a temperature that denatures the double-stranded DNA and allows the target sequence to be exposed, but does not denature the thermostable polymerase. Denaturation of the DNA molecule occurs as the hydrogen bonds between complementary bases weaken at this high temperature. The reaction is then cooled to 50-65ºC, a temperature defined by the base content of the primers and optimized to allow hybridization of only the primers to the specific target. This temperature optimization is critical to the specificity and robustness of the protocol, and is often 2-3ºC lower than the melting (denaturation) temperature of the primers. If poorly designed, the primers may bind to each other instead of the target at this point (if their sequences are complementary), or may bind incompletely (one primer anneals properly, but the GC content of the other prevents annealing at the same temperature), which would prevent accumulation of fragments of the correct size in numbers easily viewed in a agarose gel. Also during this step, the polymerase binds and begins elongation as the mixture is heated to the optimum temperature for the polymerase. This temperature depends on the enzymatic activity of the polymerase used, but is generally around 72ºC, the optimum temperature for Taq polymerization of the new strand of DNA. Taq

polymerase was originally derived from a thermophilic bacterium discovered in hot springs at Yellowstone National Park, *Thermus aquaticus* (Brock, 1969). Synthesis of the new DNA strand occurs by the addition of dNTPs complementary to the target strand in the 5' to 3' direction, polymerizing phosphate groups of the dNPTs to the exposed hydroxyl groups of the template DNA. The length of time at this temperature depends on the polymerization speed of the enzyme and the length of the target, often calculated at 1,000 bases per minute. These three temperatures are repeated for upwards of 35 cycles, followed by a final elongation cycle at around 72ºC to complete the extension of any remaining singled-stranded DNA fragments.

Designing new PCR protocols

Combining the sequence data available through sources such as NCBI with the use of oligonucleotides designed to complement specific regions of the target genome, plant pathologists can design primers to amplify specific organisms or groups of organisms from plant disease samples. The specificity of the amplification can be modulated by the choice of target region, annealing temperature of the primers, concentration or presence of salts and other chemicals in the PCR mix, and competition for amplification by nucleic acids in the complex mixture of microorganism and plant genomic DNA in plant diagnostic samples. Development of a PCR detection protocol for diagnostic use should include consideration of the specificity desired, anticipated copy number of the target sequence per cell, ease of detection in complex mixtures of plant and organism compounds, and cross-detection of host DNA or that of other commonly-associated organisms.

The specificity of a target depends on whether one wants to detect a genus, species, or even subspecies, and if the organism(s) genome contains regions that can

accommodate the amplification and differentiation of such a target. Highly conserved regions of a genome can be targeted across species within a genus, across genera within families, or even across phyla. Conserved sequences within the 16SrRNA gene in bacteria are often used in this manner (Michael Innis, 1990). Conversely, a highly-specific target can be developed that is based on a highly-variable portion of the genome such as those within the Internal Transcribed Spacer region (ITS) in fungi. Sensitivity of the conventional PCR amplification can be increased by combining conserved and variable targets in nested PCR.

Application of PCR in identification of pathogens

PCR is rapid, cost effective, and the use of genus-specific primers would enable NPDN laboratories to detect a large number of pathogenic organisms including new or undescribed viruses, bacteria, fungi, and nematodes. Genus-specific PCR assays can detect a large number of known organisms and can even assist with the detection of new ones. Results of PCR from samples give sufficient information (presence-absence) for a diagnosis to be sent to the grower who can take appropriate action and can be incorporated into research for population studies or used as the basis of additional phytosanitary testing. Amplified PCR products from the sample also can be used for identification to species or even subspecies if sequenced or used in a nested procedure. Sequences can be obtained in as little as two days and can be compared to known sequences in GenBank®. When combined with PCR of a known positive control, the presence of bands of the same size constitutes a positive result that can be combined with other test results to draw conclusions about the identity of an organism, even one that is not known to be present in that location.

PCR assays are very sensitive and false positives are rare when the assays are performed correctly. False negatives are one pitfall that can be avoided through the use of internal controls and extraction of high-quality DNA from the correct plant or soil sample. The risk of false positives and negatives can be reduced through the incorporation of controls such as known positive control DNA run in tandem to the target DNA as well as internal controls specific to the PCR reaction.

Barriers to incorporation of PCR in the diagnostic laboratory

Sixty years after Watson and Crick's work, and twenty years after Mullis', we no longer discuss at great length the molecular mechanism of PCR; we tend to simply use the acronym to name one of a set of methods used during scientific investigation. The seismic scientific shift caused by this discovery notwithstanding, today's use of PCR is routine and ubiquitous in research. Its potential for diagnostic use is changing the nature of regulatory and extension diagnosis, but there are hurdles to overcome to encourage its application in most clinics (Vincelli, 2008). These hurdles are diminishing as more organism sequences are published in GenBank®, primer sets and amplification protocols are developed in research laboratories and published, and nucleic acid extraction is made easier by the commercial availability of kits and reagents. Current issues remain, though, and include the lack of time for a diagnostician to search the literature, compare protocols, troubleshoot and optimize those protocols for their target organism or laboratory setup. Additionally, there is a need to standardize methods across laboratories to encourage trust in diagnostic results, and diagnosticians need training to refresh their molecular skill set. Of course, the investment in equipment, consumables, and training may be out of range for smaller laboratories or those without adequate support beyond sample fees or state/federal funding. For instance, some

smaller laboratories in the Caribbean Regional Diagnostic Network may find it easier to send samples to Florida, Puerto Rico, or the Dominican Republic, where labs have been equipped with PCR capability.

Valid PCR controls and standards. DNA-based tests run without appropriate positive controls are invalid, so laboratories using these tools must maintain known reference cultures and DNA collections that serve as positive controls. Maintaining such growing collections constrains the expansion of the use of DNA-based plant diagnostics, due to the demands of acquiring the appropriate permits, identifying appropriate sources of cultures, and maintaining viable cultures to use as positive controls/references.

PCR combined with bacterial clones presents a solution to one of the problems facing diagnostic clinics – maintenance of a positive control. Diagnostically-relevant parts of pathogen genomes can easily be inserted into plasmids that can be amplified in *E. coli* bacteria (cloning/transformation). When the cloned regions of the pathogen genome contain the binding sites of the primers, they can function as immortal positive controls that pose no risk of spreading the pathogen since the cloned amplicon is not infectious. Cultures of *E. coli* that contain the control DNA can be stored for years and are reactivated with standard equipment present in diagnostic labs. Large amounts of DNA can be quickly produced and purified for use as positive controls as needed. Amplicons cloned in *E. coli* are quickly and easily grown and harvested by miniprep kits in the laboratory, resulting in large quantities of high-quality template DNA for PCR.

While *E. coli* strains could be distributed among laboratories with appropriate permits, and maintenance of *E. coli* strains can be standardized regardless of the

amplicon segment, shipment of any active culture requires permits and precautions. However, the plasmid DNA is extremely stable and easily amplified, and could be shared with other laboratories as simple extracted DNA in lyophilized or cold-stored form. In addition, the use of competent, commercial *E. coli* plasmids allows for elucidation of positive results for a new pathogen versus detection of contaminating control DNA; the cloned plasmids contain known sequences that can be detected with PCR and proven by sequencing or restriction enzyme digestion, reducing the concern regarding contamination of testing laboratories with control DNA. However, quality control of the bacterial clone is an issue that will need to be addressed; contamination of the clone culture is possible, and detection of the contamination may be difficult in a diagnostic lab that uses the clone only a few times per year.

Examples of this system of positive control clones exist, including one which was utilized for detection of the soybean rust causal agent before the pathogen was widespread in the US. When a Taqman®-based real-time PCR technique (Frederick, Snyder, Peterson, & Bonde, 2002) became available for identification of *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust, implementation of the test depended on having genomic DNA for that species as well as the closely related species, *P. meibomiae.* Supplies of genomic DNA were limited to a few regulatory and research laboratories. Therefore Vincelli et al. successfully cloned the amplicons for both species (using Promega's pGEM®-T Easy Vector System transformed into *E. coli* strain JM109), determined that the miniprep DNA reacted very well in PCR tests, and had their methods and results peer-reviewed by the relevant researchers and regulators. With USDA approval, these clones have been made available throughout

the National Plant Diagnostic Network, along with instructions for their storage as well as use in PCR assays. This service has been considered very valuable by recipients of these materials, and it serves as a model for how cloning, testing, and distributing amplicon to serve as positive controls will enhance the application of powerful DNA-based tools for pathogen detection.

Matrix matters: Host and pathogen attributes influence DNA extraction and PCR

Variables such as sample particle size, presence of polysaccharides, etc., determine nucleic acid extraction efficiency (Tebbe, 1993) (Klein, 1997) (Vanysacker, 2010) (Santos, 2010) (Hyeon, 2010). Although we cannot hope to develop a protocol for every host-pathogen combination, we may be able to develop some guidelines for extraction from recalcitrant host tissue or pathogen matrices.

CTAB- and EDTA-based protocols have been the extraction method of choice in research and certification laboratories in the past and continue to be utilized, but these protocols require hazardous substances and time-consuming procedures. Additionally, countless versions have been published, making it difficult to pick just one for use in a diagnostic laboratory. However, when applied correctly and consistently, these methods are inexpensive and result in a clean extraction.

One way of standardizing DNA extraction while minimizing exposure to hazardous materials and decreasing hands-on time is through the use of commercial nucleic acid extraction kits. Most NPDN diagnostic laboratories prefer to utilize these kits as a way to minimize mistakes and standardize methods. In addition, the kits last a long time, most components can be stored at room temperature, and the instructions are easy to understand. However, we have found that many of these kits are of limited use in extraction of DNA from certain host and pathogen matrices, such as those with

high phenolic or polysaccharide content. For example, three commonly-used DNA extraction kits yielded no amplifiable DNA when used with pure cultures or woody plant tissue infected with *Raffaelea lauricola*, the causal agent of laurel wilt. Furthermore, it may be difficult to recover genomic DNA from fungal propagules with refractory cell walls, such as oospores or melanized conidia, substantially reducing detection sensitivity of pathogens and even resulting in false negatives (Zhang, 2010). Viscosity due to the presence of polysaccharides decreases the accuracy of pipetting. Polysaccharides comingle with DNA, get caught in spin-column filters, and may inhibit PCR. Centrifugation may help draw the polysaccharides away from the supernatant (DNA), but will decrease the total amount of DNA available for amplification, and may shear the DNA.

Simple modifications of the procedure, such as dilution of the extract and/or lengthening the time the sample is in extraction buffer, might enhance the utility of these kits. There is plenty of research to indicate this is already possible (Jurick, et al., 2007) (Harmon, Dunkle, & Latin, 2003). However, dilution of the nucleic acid can affect PCR efficiency, and if the amount of sample tissue is minimal, or the pathogen is minimally present in the sample tissue, dilution may produce false negatives with PCR.

Once extracted, nucleic acid amplification may be inhibited by the presence of compounds that interfere with DNA denaturation or the function of the polymerase. The presence of phenolic compounds, which bind to DNA after cell lysis and interfere with DNA amplification, is common in many woody plant hosts. Phenolic compounds from the host material or the extraction process may interfere with the activity of the polymerase. Stabilizing compounds added to reconstituted DNA (TE – Tris-EDTA) can

inhibit PCR, but long-term storage of DNA without them (in sterile distilled water) is unlikely to yield a high-quality product. Additionally, polymerases are sensitive to salts, especially magnesium, and host materials with higher or lower amounts of this specific salt may affect the ensuing PCR reaction.

Conventional PCR: Approachable and Affordable

The current trend in molecular identification of organisms is towards real-time, or quantitative PCR (qPCR). We intend to develop and adapt procedures for conventional PCR because while nearly every diagnostic laboratory in the US now has access to a thermocycler and gel electrophoresis and documentation for conventional PCR, only a few have access to qPCR. The sensitivity of qPCR is offset by the comparatively costly investment in equipment, supplies, maintenance, and training necessitated by this method.

Sensitivity of conventional PCR can be increased with the use of nested and multiplex PCR protocols. Nested PCR employs universal primers for putative detection of several or all members of a genus, such as *Phytoplasma*. The high sensitivity associated with the nested technique is preferable for woody tissues, because detection using single-round PCR in infected woody hosts can fail. A positive reaction by this PCR technique is indicative of the presence of phytoplasmas. Direct sequencing of the amplicon is only necessary if it is desirable to identify the species of *Phytoplasma* present.

Whether increased in sensitivity by nested or multiplex protocols or not, conventional PCR results are generally adequate for diagnostic laboratory clientele. Over the past four years, the UF Plant Disease Clinic (PDC) has been trialing PCR protocols for the detection of unculturable pathogens such as *Xylella fastidiosa*, the

phytoplasma that causes Lethal Yellowing of Palm (*Candidatus* Phytoplasma palmae), and *Blueberry red ringspot soymovirus*. These protocols were developed in research laboratories and we have adapted some without modification (*X. fastidiosa*) while others have needed some refinement of the nucleic acid extraction steps. Working with researchers specializing in the relevant pathogen or host groups has enabled us to develop simple work instructions for the protocols and add the tests to the menu of services available to our extension clientele.

Regulation and quality controls

Plant diagnostic and research laboratory work is subject to regulations at the university, state, and federal level. At the federal level, the USDA Animal and Plant Health Inspection Service (APHIS) Plant Protection and Quarantine (PPQ) division is authorized by the Plant Protection and Honeybee Acts to regulate by permit the movement of plants, soil, and any organisms that the plants or soil might harbor. Additionally, work on specific microorganisms requires permits that regulate the shipment, receipt, storage, and destruction of the organism and may also define the parameters of the research itself (release, genetic transformation, etc.).

NPDN diagnostic laboratories may receive diagnostic samples from outside their state boundaries, and so are required to obtain a permit to receive such samples. The PPQ 526 permit is “required for the importation, interstate movement and environmental release of plant pests (plant feeding insects, mites, snails, slugs, and plant pathogenic bacteria, viruses, fungi, etc.), biological control organisms of plant pests and weeds, bees, parasitic plants and federally listed noxious weeds” (USDA-APHIS, 2010). Diagnostic laboratories are authorized to receive plant or soil samples for diagnostic use only, so at the point of diagnosis, the sample must be destroyed according to the

protocol outlined in the permit (often by autoclaving). Laboratories that wish to maintain cultures of organisms from out of state for research or reference use must apply for permits to maintain those cultures. Lab-reared cultures (such as the bacterial clones in this proposed research) are governed by similar permitting rules, so the maintenance of those cultures can only be at laboratories authorized to receive and maintain them. For the purposes of this research, all the bacterial clones will be maintained by the main lab, and only extracted DNA will be sent to the participating laboratories. No permit is required for the exchange of extracted DNA. Relevant regulations pertaining to the maintenance of the clones include 1) *Any genetically engineered organism composed of DNA or RNA sequences, organelles, plasmids, parts, copies, and/or analogs, of or from any of the groups of organisms listed below shall be deemed a regulated article if it also meets the definition of plant pest in § 340.1, and 2)Excluded are recipient microorganisms which are not plant pests and which have resulted from the addition of genetic material from a donor organism where the material is well characterized and contains only non-coding regulatory regions.*

Plant pathogens selected for this project

The NPDN SOPs were written to guide diagnosticians in the detection of high-impact plant pathogens and pests. The complete list of SOPs is available at www.npdn.org. We have focused on a select number of these pathogens in order to develop a system that can continue beyond the grant funding period. We worked with both bacteria and fungi to ensure a robust system applicable to future work. Not all of the pathogens were used for all of the objectives, but we used those that best tested the system for each step of the process.

Raffaellea lauricola. Laurel wilt is a new disease in the southeastern US (Harrington, 2008). The NPDN SOP is based on work by Harrington (Harrington, 2008) and Smith (Dreaden T. A., 2009), (Dreaden T. D., 2013). First discovered in Georgia in 2002, the disease has since spread to more than 52 counties from South Carolina to Florida. *R. lauricola* is vectored by the nonnative ambrosia beetle, *Xyleborus glabratus*. While the vector was known to science the pathogen is new. *R. lauricola* is an asexual relative of the well-known genus of fungal tree pathogens, *Ophiostoma*. There have been no reports of laurel wilt in native lauraceous plants that the beetle infests in its native Asian homeland, but to date several American members of the *Lauraceae* have been susceptible. In particular redbay (*Persea borbonia*) has been decimated in much of its natural range. *R. lauricola* damages the tree by blocking the flow of water through the tree and causing wilt. Prior to 2006, laurel wilt research focused on the disease's impact in native ecosystems in the southeastern US. During this work a commercial crop in the *Lauraceae*, avocado (*Persea americana*), was discovered to be susceptible. Subsequent work has shown that *X. glabratus* is attracted to and is an effective vector of *R. lauricola* to healthy avocado in the field and greenhouse. Breeding efforts for disease resistance are in process, but there are currently no complete management solutions. Early detection allows for tracking of the disease and removal of infected trees to slow disease spread. It is likely that this disease will continue to spread westward and northward from the current locations to the extent of the host's natural range and early detection will be key.

***Candidatus* Liberibacter asiaticus.** *Ca*. L. asiaticus is a phloem-limited bacterial pathogen causing huanglongbing (formerly known as citrus greening). The

NPDN SOP is based on work by Jagoueix and Li (Jagoueix, 1996) (Li, 2006). The pathogens that cause huanglongbing can be found on nearly every continent, causing crop and tree loss (Graca, 1991). The disease has been reported since the early 1900s. Crop losses approach 100% where the pathogen and vector are endemic. Three species of the pathogen are known today, none of which can be cultured with current methods. Molecular techniques have differentiated the three species of *Ca*. Liberibacter (Li, 2006). *Ca*. L. asiaticus is vectored by the Asian citrus psyllid *(Diaphorina citri)* and is found primarily in Asia and limited areas in North America. *Ca*. L. africanus is vectored by the African citrus psyllid (*Trioza erytreae*) and is found primarily in Africa, and a third species was reported in South America, *Ca*. L. americanus (Teixeira, 2005). All three species cause typical greening symptoms and all are vectored by psyllids. All *Citrus* species and cultivars are susceptible, although some are reportedly more tolerant of the infection. Those cultivars and species with some tolerance are still important as disease reservoirs. Other genera reported as hosts include rutaceous plants such as kumquat (*Fortunella* sp.) and *Murraya* spp. In Florida and other southern states, *Murraya* spp. are important in movement of the disease and the vector, as psyllids find *Murraya* spp. especially attractive.

Phytophthora ramorum. *P. ramorum* is an oomycete regulated due to its destructive nature in western coastal forests and ease of asymptomatic plant transport. The NPDN SOP was based on work by Hayden et al. (Hayden, 2004). *P. ramorum* is an oomycete plant pathogen that causes Sudden Oak Death (SOD) and Ramorum Blight. SOD is a forest disease that results in dieback of several tree species in California and Oregon. Ramorum Blight is a non-fatal disease that affects the leaves

and twigs of foliar hosts, particularly found in understory plants. *P. ramorum* prefers cool and wet natural climates. In California, coastal evergreen forests and tanoak/redwood forests within the fog belt are the primary habitat. Nurseries outside of this area often mimic these conditions and allow *P. ramorum* to flourish far outside of its natural environment. The possible impact of uncontrolled SOD/Ramorum Blight includes a change in species composition in forests and therefore in ecosystem homeostasis. Currently, there are several published regulations controlling the movement of plant material from infected counties in California, Oregon, and Washington. National surveys are conducted annually and research is being performed by various private and public institutions on the detection, transmission, and treatment of SOD. There is no chemical treatment available to eliminate *P. ramorum* in nursery stock at this time. There is, however, a prophylactic treatment available for use immediately following the onset of symptoms or after detection. Control of this disease relies heavily upon early detection and proper disposal of infected plant material.

Xylella fastidiosa. *X. fastidiosa* causes bacterial scorch of many hosts and is becoming an increasingly important production issue in blueberries and woody ornamentals in Florida. The bacterium is extremely difficult to culture and current ELISA-based detection technologies frequently result in false positives due to environmental contaminants (pollen, dust) or false negatives due to the low quantity and erratic distribution of the pathogen within the host. Confirmation of the disease in nurseries and fields allows for the removal of infected hosts, reducing inoculum for this vector-borne pathogen. Management of the vector and removal of infected plant material are the only management options currently available.

***Clavibacter michiganensis* subsp. *michiganensis*.** This gram-positive bacterial pathogen causes bacterial canker in tomato. The pathogen may be introduced to the greenhouse or field in symptomatic transplants. Mature plants may wilt and die, especially when water and nutrient needs are highest, such as during fruit development. Although it is not difficult to culture, confirmation of the species and subspecies is important and is difficult via culturing alone. Early detection in transplants and grafting operations is important in disease management.

Phytophthora cinnamomi. *P cinnamomi* is a non-regulated oomycete ubiquitous in woody ornamentals in the southeast US. The pathogen is not native to the US, but was introduced and is now endemic. The pathogen survives in surface water and plant material and morphological differentiation between *P. cinnamomi* and *P. ramorum* is difficult. Current ELISA-based methods do not distinguish between species within the genus. The host list for *P. ramorum* includes hundreds of species, and detection of the genus in listed plant material requires additional regulatory testing for *P. ramorum*.

Selection of the specific pathogens listed above was based on several factors including 1) economic or environmental impact if/where the pathogen becomes established, 2) availability of published diagnostic protocols that can be adapted for diagnostic laboratory use, 3) difficulty of culturing the pathogen or identifying the pathogen to species from culture, 4) the desire to work with as many types of pathogens as possible in order to ensure a robust system for future work, 5) the frequency of false positives or negatives in the current diagnostic protocol, such as those that utilize ELISA, and 6) the lack of distributed diagnostic capacity for the pathogens.

Objectives

Research activities have focused on the production of high-quality, standardized controls and the recommendation of efficient methods for DNA extraction. Research components included investigation of 1) commercially-available options for obtaining adequate quantities of amplifiable DNA from plant tissues, 2) PCR-inhibiting compounds in plant host material, 3) the development of positive control DNA clones for multiple pathogen types, and 4) the comparative analysis of the recommended extraction methods across five diagnostic laboratories.

Commercially-available Options for Obtaining Adequate Quantities of Amplifiable DNA from Plant Tissues Free of PCR-inhibiting Compounds

Plant compounds such as lignin, humic acid, starches, phenols, latex, and others are often implicated in the inhibition of nucleic acid extraction and/or PCR. Given the wide range of host material confronted by diagnostic laboratories, it would be useful to define a single extraction protocol that would eliminate inhibitory compounds. This will improve the robustness of current PCR protocols, and increase the confidence of diagnostic personnel in the use of PCR as a diagnostic tool.

We investigated humic acid, a plant derivative that contains several phenolic compounds. We chose plant tissue from a variety of recalcitrant host tissues, including wood, roots, citrus midribs, herbaceous plants with copious latex sap, and tuberous roots. We tested commercially-available DNA extraction kits that are reported to extract from high-phenolic tissues. We analyzed aliquots of post-extraction kit DNA to determine the presence of inhibiting factors prior to PCR. Additionally, we spiked healthy plant samples with known quantities of humic acid to determine the level of PCR

inhibition, and tested whether we could dilute the sample enough to avoid inhibition, but not dilute the nucleic acid beyond detection.

Phytophthora ramorum. PCR protocols for both conventional and real-time PCR have been developed for this pathogen and these protocols were utilized for the development of a double-insert PCR clone. The extraction procedure is set by USDA-APHIS, so we incorporated the recommended Qiagen kit into our tests.

***Candidatus* Liberibacter asiaticus**. PCR protocols for both conventional and real-time PCR (Li, 2006) have been developed for this pathogen and these were utilized for the development of a double-insert PCR clone. We tested DNA extraction methods on citrus midribs, the plant tissue usually targeted for detection of this pathogen.

Raffaelea lauricola. The PCR amplicons for each of the two assays were incorporated into a double-amplicon clone. DNA extraction methods were tested on woody host tissue, which is anecdotally reported by diagnosticians to be high in phenolic compounds.

Xylella fastidiosa. A clone for this pathogen was developed previously. We included it in our studies to compare an established clone with the quality and utility of the newly-developed cones for other pathogens. DNA extraction methods were tested on blueberry midribs, which are anecdotally reported to be high in phenolic compounds.

***Clavibacter michiganensis* subsp. *michiganensis*.** PCR protocols for conventional PCR were utilized for the development of the PCR clone. Extraction of DNA from tomato is not generally problematic in the diagnostic laboratory, but the pathogen is important and the DNA extraction protocol and positive control were requested by diagnosticians.

Phytophthora cinnamomi. The PCR amplicon for a genus-level PCR assay was incorporated into a clone. DNA extraction methods were tested on high-phenolic host tissue.

Production of Positive DNA Clones

Supplies of genomic DNA for each of our selected pathogens are limited, but without appropriate positive controls, PCR protocols are unvalidated for diagnostics. We obtained the genomic DNA from the appropriate collaborator (*P. ramorum* and *Ca.* L. asiaticus from USDA-APHIS-SMMBL, *R. lauricola* from J. Smith, UF; *C. michiganensis* subsp*. michiganensis* from PDC samples, and *P. cinnamomi* from P. Harmon, UF). We then used the primers from each protocol to amplify the appropriate amplicon, cloned the amplicon using commercial *E. coli* vector systems, tested the cloned amplicon to ensure it reacted appropriately to subsequent PCR tests, and sequenced the cloned amplicon to ensure quality. Clones for *P. cinnamomi, P. ramorum*, '*Ca*. L. asiaticus', *R. lauricola* and *C. michiganensis* subsp. *michiganensis* were developed and tested in the Harmon laboratory. A clone for *X. fastidiosa* was provided by the Jones laboratory at UF.

Comparative Analysis of Protocols

NPDN diagnostic laboratories at the University of Florida, Cornell University, Louisiana State University AgCenter, Purdue University, and Texas A&M University comparatively analyzed the selected extraction protocols and positive controls. Collaborators at these institutions used their own extraction protocols alongside the recommended extraction kits, and tested the PCR positive control bacterial clones. The Project Director supplied each laboratory with frozen plant tissue and clone to standardize the substrate across the laboratories. Collaborating laboratories compared

ease of use, efficiency, expense, and accuracy of results (true positive and negative results versus false positive and negative results). DNA was assayed via spectrophotometry for quantity and quality post-extraction and post-dilution.

Summary

Diagnosticians frequently encounter challenging samples; organisms may be difficult to culture, difficult to differentiate, difficult to speciate, and difficult to establish pathogenicity. PCR, as one of multiple identification methods utilized, can be applied to increase specificity of identifications, and increase detection of unculturable or recalcitrant organisms. Application of this method requires some standardization and validation to ensure credible, reliable, accurate, and precise identification. Further discussion of credibility, reliability, accuracy, and precision appears in the following chapter.

The research proposed aims to remove some barriers to the use of PCR as a diagnostic tool by recommending guidelines for method development, recommending standard kits that have been shown to reduce PCR inhibitors, and increasing the availability of positive controls. Our goal is to increase the use of PCR as an affordable, valid method when appropriate, enabling efficient and appropriate identification in diagnostic laboratories.

Table 1-1. SPDN diagnostician survey regarding PCR and diagnostic tools. N=13.

Would you attend a PCR workshop to obtain and learn how to use new protocols and reagents?	Would you use PCR more often if supplied with clear protocols and positive controls?	Do you have access to appropriate equipment and reagents to perform PCR?
Yes = 85%	Yes = 85%	Yes = 100%

CHAPTER 2
FACILITATING PCR SUCCESS IN THE PLANT DIAGNOSTIC LABORATORY VIA EFFICIENT EXTRACTION AND ACCESS TO POSITIVE CONTROLS

Background

Regulatory diagnoses require specific identification that increasingly can be addressed with PCR, and even non-regulatory demand for specific, accurate, and timely diagnosis is increasing. A diagnostic PCR protocol must be adequately described and should be validated through testing in multiple, appropriate diagnostic laboratories. Additionally, development, dissemination, and recommendations for use of reagents and appropriate controls should be included in a valid test. We describe a set of guidelines for developing and testing appropriate extraction methods and PCR positive controls for use in plant diagnostic laboratories, based on the successful use of DNA extraction kits and cloned amplicons as PCR positive controls. Our goal was to standardize nucleic acid extraction, determine whether spectrophotometric analysis of kit-extracted DNA correlated to amplification, and reduce false positives and false negatives through the use of cloned positive controls.

Spectrophotometric analysis of total quantity or protein contamination of extracted DNA did not correlate to amplification. However, high A260/230 ratios, which indicate a low level of phenolic contamination in extracted DNA (Wallis, 2012), correlated to amplification. Tests of individual labs indicated a significant level of variability by DNA extraction kit, and different kits extracted amplifiable DNA from various host tissues in a significantly different manner. We identified two commercial extraction kits, from a test of five kits, which standardize a reduced number of steps and total time required to extract amplifiable DNA from recalcitrant host tissues. Finally, we developed positive controls for several pathogens of interest. Combined, these findings

will save time and increase the likelihood of successful PCR tests in the diagnostic lab, which should increase the confidence in and use of PCR as a diagnostic tool.

Reliability, Accuracy, and Precision

Equipment, reagents, and pathogen-specific oligonucleotides needed for polymerase chain reaction (PCR) have become more accessible and affordable for plant diagnostic use. Many competitive funding programs have encouraged and supported research into diagnostic method development and the extension of new methods to university and regulatory laboratories. Diagnosticians have attempted to implement the developed research assays to meet an increased demand for DNA-based, specific, pathogen identification in laboratories around the world. However, diagnosticians have encountered challenges adapting research protocols to be reliable, accurate, and precise for diagnostic purposes.

Reliability

Broadly speaking, reliability is the likelihood of getting an interpretable result, regardless of precision or accuracy of the test. Whether the result is affirmative or negative, it is interpretable when appropriate positive and negative controls produce expected results.

Accuracy

Accuracy can be thought of as the likelihood that the protocol returns the correct result. An accurate protocol gives a positive result when used on the specific pathogen that the test was designed to detect, and gives a negative result on everything else.

Precision

Precision is a measure of reproducibility across labs and time. A precise protocol may not give the correct result but will give the same result when used repeatedly or by different diagnosticians on the same organism.

Specificity

A diagnosis may require a different level of pathogen identification specificity depending on the client, and management or regulatory implications of the detection. A genus-level identification may be sufficient to diagnose and provide recommendations for management of routine diseases such as Phytophthora root rot. When a diagnosis will be used as the basis for regulatory action, identification to species level or better is needed as is the case with *Phytophthora ramorum*. As specificity of a protocol increases, the breadth of organisms detected decreases. As specificity increases, the need for additional tests to confirm an identification, and associated costs, also increases (a negative result for a species-specific test means a diagnostician will need to run another test to identify the organism). Specificity of a given protocol is independent of its reliability, accuracy, or precision.

Incorporating New Tests

While specificity is generally determined and reported when an identification protocol is published, accuracy, precision, and reliability may vary by diagnostic lab, and are not always static. It is important that a diagnostician know the reliability, accuracy, and precision of a given protocol so that they can determine whether the protocol is appropriate for a sample and client. A protocol adopted in a diagnostic lab must be practiced with some regularity to reduce error, and resources are finite in a diagnostic

lab, so a protocol is likely to be incorporated only if is relevant to a reasonable proportion of the samples routinely encountered in a given lab.

Additional barriers to the adoption of PCR assays include training, access to controls, and inhibitors or other confounding aspects to the PCR process itself.

PCR assays are not valid without appropriate positive and negative controls, and inhibitors to the extraction and amplification processes are well-documented (Teebe, 1993) (Hyeon, 2010) (Haudenshield, 2011). Spectrophotometric analyses of A260/A280 and A260/A230 ratios and DNA concentration are often used to analyze purified DNA for PCR inhibitors and to predict the probability of successful amplification in research settings. However, the value of these analyses on crude DNA extracts common in diagnostic use is not well documented. We investigated whether there was a correlation between amplification and DNA concentration and purity, as measured by a Nanodrop spectrophotometer (Thermoscientific, Wilmington DE), to determine if this could be a cost-saving step prior to investing in the reagents required for a PCR run. Additionally, we investigated the effect of humic acid, a PCR inhibitor common in necrotic plant tissue.

Positive Controls

The nature of a diagnostic laboratory often precludes storing and managing large culture collections, so diagnosticians may find it difficult to obtain DNA to use for controls unless a researcher or regulatory agency can supply the DNA on an ongoing basis. Cloning PCR amplicons into bacterial plasmids creates a perpetual supply of positive controls. Although development of positive controls in this manner is not a new idea (Rosenstraus, 1998) (Haudenshield, 2011) (Minsavage, 1994), we see potential in this system as a mechanism to fill the demand for positive controls in plant diagnostic

laboratories worldwide. When diluted appropriately and included in parallel samples, the plasmid DNA can serve as both a control for the protocol and for the reaction since the control amplifies with identical primers and conditions as the test protocol. Reference cultures and isolates cannot be shipped across state borders without permits, but DNA can be exchanged without permits. Additionally, DNA can be stabilized by shipping on ice or in a lyophilized state, and storage is similarly easy. Stringent quality controls need to be in place to ensure the value of the DNA as a control. On a small scale, this is difficult for any one laboratory to keep up with, but it is viable if the storage of the plasmids is curated in a larger collection by a laboratory designated for this purpose.

We investigated the potential application of cloned amplicons for use in diagnostic laboratories. PCR targets were chosen for the cloning process based on four criteria: 1) the pathogen is not endemic, and early detection could lead to effective management, 2) the pathogen is difficult to culture, or difficult to differentiate in culture, 3) the protocol is one that NPDN diagnostic laboratories have been asked to run for their clientele, and 4) a conventional PCR protocol has been developed, published, and validated through use. However, once the system is in place to manage the quality and availability of the plasmid DNA, this system could conceivably be used for any pathogen or other organism of interest. This would allow diagnosticians to simply order the controls as they would controls for any other technology such as ELISA, encouraging the adoption of PCR as a standard tool in plant diagnostic laboratories worldwide.

Objectives

Our objectives were to 1) identify a commercially-available DNA extraction procedure that was quick and produced enough amplifiable DNA for diagnostic use, 2)

determine the utility of spectrophotometric readings in predicting successful amplification, 3) determine the influence of humic acid on amplification, and 4) develop and test cloned PCR amplicons for their use as positive controls. Within these main objectives, we also investigated the variability between laboratories, and the reliability, precision, and accuracy of the PCR protocols when applied to artificial diagnostic samples.

Materials and Methods

Comparison of PCR Capacity in Five Laboratories via PCR Amplification of 18S rDNA from Healthy Plant Tissue.

The five laboratories involved were the plant disease diagnostic laboratories at Cornell University, Louisiana State University AgCenter, Purdue University, Texas A&M University, and University of Florida. To prepare the laboratories for running the pathogen-specific PCR tests, and to assess if any labs were anomalous in their capacity to perform PCR, staff at all of the laboratories were asked to perform three different DNA extraction protocols, followed by a single PCR protocol to detect plant DNA via 18S universal primers NS1-NS2 (White, 1990).

DNA extraction kits and PCR protocols

Two kits were standardized across all five labs (QIAGEN Stool DNA extraction kit and Zymo Research Corp. Plant mini kit); the individual lab chose the third extraction kit to use, based on their familiarity with the kit or desire to try it (table 2-1). Each lab was provided with the PCR primer sequences, thermocycler settings, and approximate expected band size (table 2-3).

Preparation of samples and PCR

Each lab was provided with a set of frozen homogenized plant samples from the list of eight recalcitrant plants tissues below:

1. Azalea (*Rhododendron* sp.) leaves
2. Blueberry (*Vaccinium* hybrid) midribs
3. Blueberry (*Vaccinium* hybrid) roots
4. Citrus (*Citrus maxima*) midribs
5. Euphorbia (*Jatropha* sp.) leaves
6. Potato (*Solanum tuberosum*) tubers
7. Swampbay (*Persea palustris*) cambium
8. Tomato (*Lycopersicon esculentum*) stems

For each lab, each tissue type was represented by 18 sample tubes; three for tissue extractions by each of three kits, times three kits, repeated once (Table 2-7). The plant tissue DNA was extracted as per the manufacturer's instructions, then stored frozen until analyzed with spectrophotometry and PCR. PCR runs included a nontemplate negative control (mastermix of primers, polymerase, dNTPs, and buffer, without plant DNA). The plant tissue itself acted as the positive control; if there was plant tissue, the 18S primers should amplify the DNA with the universal primers unless the kits had not removed the enough of the inhibitors (Vanysacker, 2010) (Klein, 1997) or the DNA was trapped in the spin column filters by commingled polysaccharides.

Data reporting and equipment

Each lab reported three spectrophotometric values (DNA concentration (ng/µl), A260/A280 and A260/A230) as well as amplification (as depicted in images of UV-illuminated agarose gels). Amplification was originally reported as 0, 0.5, and 1, where a 0 represented no visible band, a 0.5 represented a very faint band that would normally require a second PCR run to confirm, and a 1 represented a clearly-visible band. The amplification values were updated to binary 0 or 1 for statistical analysis; the 0.5 values

were all corrected to 1 values and reported as corrected amplification, indicating the presence of a band, even if only faintly visible. The proportion of samples that produced the expected size amplicon was reported as percent correct amplification. Negative values for DNA concentration (ng/µl), A260/280, and A/260/230 were corrected to 0.001 to allow for statistical analysis, and were reported as corrected concentration(ng/µl), corrected 260/280, and corrected 260/230.

Labs purchased their own polymerase, primers, and mastermix reagents. All labs used a Nanodrop spectrophotometer, but the PCR thermocyclers varied according to existing equipment available in the laboratories (table 2-2).

Statistical analysis

Multivariate analysis (SAS, Version 9.3 and JMP version 10, SAS Institute, Cary, NC; IFAS Statistics Department, UF, Gainesville, FL) was performed on the data from all five laboratories to determine the effect of laboratory, DNA extraction kit, and host tissue on amplification. A logistic regression of binomial data was performed with the GLIMMIX procedure, and statistically significant differences were identified with the LSMEANS statement. *p*-values were adjusted for multiple comparisons and unbalanced data ($\alpha=0.05$) with the Tukey-Kramer option.

Determination of the Effect of DNA Extraction Kit on Neutralization of Humic Acid

DNA extraction kits and PCR protocol

Two kits were further investigated for relative neutralization of humic acid spiked into healthy plant tissue samples (QIAGEN Stool kit and QIAGEN DNeasy Plant mini kit). PCR primer sequences, thermocycler settings, and approximate expected band size was as previously used (NS1-NS2 primers) in tests of healthy plant tissue. Humic acid concentrations were 0ppm (sterile DI water only), 100ppm, and 1000ppm.

Negative controls included a plant-tissue-only sample and the 0ppm sample in each set. Volumes of each additive, water or humic acid solution, were 200µL, per sample tube.

Preparation of samples and PCR

DNA extraction and subsequent amplification was performed on a set of frozen homogenized plant samples from the list of plants tissues below:

1. Azalea (*Rhododendron* sp.) leaves, including midribs
2. Blueberry (*Vaccinium* hybrid) leaves, including midribs
3. Tomato (*Lycopersicon esculentum*) stems

Each tissue type was represented by 20 sample tubes; one plant-tissue-only tube, plus three for each of the three concentrations of humic acid tissue, for each of the two kits. The experiment was repeated once. The DNA was extracted as per the manufacturer's instructions, and then stored frozen until analyzed with PCR. PCR runs included a nontemplate negative control (mastermix of primers, polymerase, dNTPs, and buffer, without plant DNA). As before, the plant tissue itself acted as the positive control.

Data reporting

Amplification, as depicted in images of UV-illuminated 1.5% agarose gels, was the final documentation for this test.

Development and Testing of Bacterial Plasmid Clones and PCR Inhibition by Humic Acid

DNA preparation

Genomic DNA was obtained from reference laboratories or prepared from pure cultures from Florida samples as follows: Genomic DNA of '*Ca*. Liberibacter asiaticus' (from known-positive plant samples) and *Phytophthora ramorum* (from pure culture) was obtained from the USDA-APHIS-SMMBL. Genomic DNA was obtained from pure

culture of *Raffaelea lauricola* from an infected redbay, grown on CSMA medium. Genomic DNA was obtained from pure culture of *Phytophthora cinnamomi* grown on PARP medium. Genomic DNA of *Clavibacter michiganensis* subsp. *michiganensis* was obtained from a pure culture obtained from a Florida tomato sample and grown on general bacterial nutrient agar. We amplified each target sequence as previously published (table 2-3) (Dreier, 1995), (Hayden, 2004), (Jagoueix, 1996), and (Dreaden, 2013). PCR amplicons were visualized on a 1.5% agarose gel to verify expected band size and quality and then were sequenced to verify the identity of the amplicon.

Cloning of plasmids

The amplicons were cloned using the Promega pGEM®-T Easy Vector System II (cat. A1380, Promega North America, Madison, WI), as directed in the manufacturer's instructions. The resulting transformants were selected, and orientation and sequence of the insert were verified by NCBI BLAST analysis of sequences (Sanger sequencing, UF-ICBR). A plasmid map of one of the clones is provided as an example (Figure 2-1). A previously-developed and tested clone for *Xylella fastidiosa* (Minsavage, 1994) was included to compare with the general performance of the new clones developed in this project. The clones (table 2-3) were assayed with the appropriate PCR protocols to ensure the cloned amplicons work as positive controls without any modification of the protocol. Bacterial clones were stored at -80°C in glycerol stocks. When needed for testing, the transformed *E. coli* cultures were grown at 25°C in LB broth (Bertani, 2004) for 24 hours, assayed with PCR, and diluted appropriately. Clones were verified via sequencing prior to shipping the controls and samples to the ring-test labs.

Ring-Testing of Positive Controls

Ring-testing is the term for inter-laboratory calibration via identical sample processing at multiple laboratories. Five NPDN laboratories tested the positive controls: Cornell University Plant Disease Diagnostic Clinic (CU PDDC), Louisiana State University AgCenter Plant Diagnostic Center (LSU PDC), Purdue University Plant and Pest Diagnostic Laboratory (PPDL), Texas A&M University Plant Disease Diagnostic Lab (TAMU PDDL), and University of Florida Plant Diagnostic Center (UF PDC).

PCR amplification of cloned pathogen DNA with species-specific primers

Staff at each of the previously-mentioned laboratories chose from at least two pathogen-host combinations from a list provided by the project director (table 2-4).

Sample preparation

To mimic extraction and amplification of pathogen DNA from host tissue, 50 µL bacterial clone culture was spiked into aliquots of appropriate host tissue. The bacterial clones (table 2-3) were grown in LB broth at 25°C for 24 hours, diluted to 10^8CFU/mL, and then killed by boiling for 10 minutes. Uninfected plant tissue appropriate for each pathogen (table 2-4) was obtained from plants grown in greenhouses at UF and observed to be disease-free. The plant tissue was processed to a fine particle size with a sample homogenizer. To prepare for each lab two sets of 12 2.0-mL tubes of plant tissue appropriate for their chosen tests, 450 µL of sample powder was aliquotted into each sample tube. 50 µL of $1X10^8$ heat-killed cells/mL of each bacterial clone culture was then added to 11 tubes of appropriate plant tissue, per 12-tube set (Table 2-8).

To assay the ability of the DNA extraction method to remove phenolic inhibitors, aliquots of 4,000 ppm humic acid (cat. 53680, Sigma-Aldrich, St. Louis, MO) were added to different tubes containing plant tissue so that the final humic acid

concentrations were 400ppm, 356ppm, 320ppm, 267ppm, 200ppm, or 133ppm. A negative control of homogenized plant tissue with humic acid but without clone was included, bringing the number of tubes to 12 per set. In order to have a blind test, sample tubes were randomized and numbered within each clone-tissue set of 12 sample tubes (Table 2-5).

Each participating lab chose at least two clone-tissue combinations as noted in Table 2-4. Each set was labeled with the host tissue to differentiate clone-tissue sets and allow for appropriate PCR protocol application. An additional tube containing 50 µL heat-killed 10^8cfu/mL clone cell suspension was included with each set of sample tubes to serve as a positive control. All samples were prepared at UF, frozen at -20°C until all sample sets were completed, then shipped on dry ice to each participating laboratory on the same day.

PCR and data collection

Each lab extracted total genomic DNA from their samples and positive control using the Qiagen QIAmp DNA Stool Mini Kit® (cat. 51504, QIAGEN, Dusseldorf, Germany), diluted the extractions 1:10 to assess whether dilution could reduce inhibition of PCR by the humic acid, processed all of the DNA with a Nanodrop spectrophotometer for DNA concentration (ng/µl) and absorbance at 230nm, 260nm, and 280nm, and prepared the samples for PCR.

Each laboratory included the positive control and a non-template negative control in each PCR run, bringing each PCR sample set to 14. PCR amplification was performed using the existing thermocyclers in each laboratory (table 2-2) to test the robustness of each protocol. Each laboratory assembled the PCR components from their normal suppliers and ran PCR, electrophoresis and gel documentation as per their

individual laboratory protocols; primers were ordered based on sequences drawn from the publications mentioned previously. Results included DNA concentration (ng/µl), A260/A230 and A260/A280 spectrophotometric ratios, and gel images documenting amplification and a yes/no conclusion by the laboratory indicating their interpretation of the gel. Final data were reported as in the first experiment: corrected amplification, corrected concentration (ng/µl), corrected 260/280, and corrected 260/230. For each clone-plant tissue combination, each laboratory processed their first set of 12 samples plus two controls through to gel documentation, then processed their second set to repeat the experiment. Samples of the positive-control amplicons sent back to UF post-test and were sequenced to verify the targets (Sanger sequencing, ICBR, Gainesville, FL).

Statistical analysis

Multivariate analysis (SAS, Version 9.3 and JMP version 10, SAS Institute, Cary, NC; IFAS Statistics Department, UF, Gainesville, FL) was performed on the amplification and spectrophotometric data from all five laboratories to determine the effect of laboratory on expected amplification of pathogen DNA. The FREQ procedure was used to produce contingency tables and perform Fisher's exact test between pairs of labs (Fisher's exact test is an analog of the Chi-square test, better suited to calculate *p*-values for comparisons of data sets with small numbers of observations and zeroes). The MULTTEST procedure with permutation option and 1000 resamples to adjust *p*-values for multiple comparisons ($\alpha=0.05$).

Results

Analysis of Three DNA Extraction Kits, Spectrophotometric Readings, and 18S Amplification of Healthy Plant Tissue Samples

The data were analyzed to determine the effect of lab, host tissue, and kit on amplification (Table 2-6). There were no differences between the two best kits, but those two kits were different from the other three kits tested. Two of the kits were not replicated, so the information regarding their reliability can only be assessed within the labs that used them. There were differences between labs and between hosts. There was a lab by kit interaction and a kit by host interaction. Neither DNA concentration (ng/µl) nor A260/280 readings correlated to amplification (P values of 0.2840 and 0.4451, respectively), but A260/230 readings did correlate to amplification (P value of 0.0034). Although amplification was observed at low and high A260/230 ratios, the mean A260/230 value was higher for amplified DNA than for DNA that had a low A260/230 value and did not amplify. High ratios (>1.7) indicative of DNA free of phenolics were positively correlated to better amplification, based on oneway analysis of corrected 260/230 by corrected amplification (P=0.0034) (Fig. 2-2).

Spectrophotometric readings overall were not reliable, as shown by comparison of the readings of the undiluted and 1:10 diluted samples. The DNA concentration reading (ng/µl) was not reduced by tenfold in the 1:10-diluted samples. For instance, the average concentration of all the undiluted samples was 7.84 ng/µL and the average of all the 1:10 diluted samples was 1.56 ng/µL. This equates to approximately a 1:5 dilution.

Extraction of amplifiable DNA was more difficult for some hosts, regardless of lab or kit (Figure 2-4). Different DNA extraction kits, as used by the different labs, had

varying effects on the amplifiability of the extracted DNA across all healthy host tissue (Figure 2-5). Both the Qiagen stool kit and the Zymo plant kit performed well across most labs and hosts. Across the two best kits an effect on amplification by lab was observed, indicating there is some variability between labs (Figure 2-6). Since the difference between the Qiagen and Zymo kits was not significant, we chose the Qiagen kit for use in subsequent method evaluation experiments due to ease of use and familiarity across NPDN labs outside our test group.

Amplification of Species-specific Targets from Clone-host-tissue-humic-acid Samples

All laboratories successfully extracted, amplified, and visualized on agarose gels the designated positive control cloned DNA from the heat-killed cell cultures. Additionally, there was overall success in amplification of the clone DNA extracted from the clone-tissue-humic acid samples. This indicates the PCR protocols are reliable across laboratories and tissues and accurate over time. Additionally, the precision of the amplification was verified for each protocol through repeated amplification of the correct size of the amplicons.

Humic acid concentrations did not have an effect on amplification when the DNA was extracted with the Qiagen Stool kit (Figure 2-3). However, when the DNA was extracted with the Qiagen DNeasy Plant mini kit, humic acid had a negative effect on amplification, especially when combined with high phenolic acid host tissue such as azalea and blueberry leaves spiked with humic acid at a final concentration of 1000ppm. Additionally, ten-fold dilution of DNeasy kit DNA extractions did not improve the rate of amplification.

We observed a host tissue-clone effect on amplification for all host-clone combinations (Figures 2-7 – 2-10) except swampbay-laurel wilt (data not shown). Percent correct amplification of both *R. lauricola* targets (CHK and IFW) from the comingled swampbay host tissue was not significantly different in any lab (P values of 0.3789 and 0.1528, respectively).

Discussion

Previous studies have reported on certain kits performing better than others at neutralizing PCR or extraction inhibitors such as phenolic or polysaccharide compounds (Klein, 1997) (Tebbe, 1993) (Vanysacker, 2010). CTAB-type extractions are often modified to accomplish just this kind of differential purification, depending on the tissue and pathogen and the purity required for the downstream application. However, kit-based extractions assist in standardization across staff and increase confidence and therefore likelihood of method adoption. Through extraction and amplification tests of eight recalcitrant healthy host tissues, we found that two kits performed admirably across most hosts and labs, indicating their use may increase the likelihood of PCR success and therefore molecular method adoption. We chose the Qiagen stool kit for use in subsequent method evaluation experiments due to ease of use and familiarity across NPDN labs outside our test group. Additionally, this kit appeared to neutralize humic acid and other phenolic compounds well in subsequent studies.

The lab by kit effect on amplification of healthy plant tissue is likely explained by the familiarity factor. Some labs were more familiar with certain kits than others, and thus were less likely to generate missteps or errors in use. The lab effect noted in Figure 2-5 indicates there is some variability between laboratories and complete

standardization, or elimination of all variability, may not be possible, even with the use of standard kits.

The lab effect during the testing of the positive control clones in healthy plant tissue samples may be explained partially by contamination. It was speculated that we may have contaminated some of the true negative samples with positive control DNA prior to sending the sets out to the testing labs. However, since other labs running the same sample sets had far fewer false positive or false negative issues with the set, we think it is more likely that some labs were better-trained and therefore less likely to make mistakes with contamination during PCR or gel setup. Any system devised to develop and supply testing panels to laboratories must have stringent quality checks in place to limit the possibility of introducing error such as this.

Although we would like to have a quick test to determine the likelihood of amplification prior to investing in the reagents and supplies, it does not appear that results of this study support the use of a Nanodrop spectrophotometer to gauge DNA concentration (ng/µl) or protein contamination of extracted DNA as a predictor of amplification. Interestingly, spectrophotometric readings of DNA concentration (ng/µl) and protein contamination did not appear to positively or negatively correspond to amplification. However, the A260/A230 reading, which corresponds to aromatic compounds such as phenolics, did correspond positively to amplification when the readings indicated the DNA was relatively free of phenolic impurities (A260/230 ratio>1.6) (Figure 2-2). Most PCR targets are in high-enough copy number to be amplifiable even when DNA concentration is very low. However, the presence of phenolic compounds can derail amplification, making the extraction process extremely

important to PCR success. Humic acid, often present in tissue being degraded by pathogens or saprophytic organisms, was incorporated into the samples to test its effect on amplification. When humic acid was added to healthy plant tissue already high in phenolic content (azalea, blueberry), successful PCR amplification was reduced when extracted with the Qiagen DNeasy kit, but not when extracted with the Qiagen Stool kit (Figure 2-3). Additionally, the high number of false negatives in the azalea leaf samples spiked with clone could also be attributed to a higher-than-average number of errors (both false positives and false negatives) at the two laboratories that processed those tissue samples. Additional experimentation has indicated that humic acid is not a problem in samples processed using the Qiagen stool kit (Figure 2-3), and high concentrations of humic acid may be neutralized adequately enough to allow amplification. The Qiagen DNeasy kit, however, did not allow amplification of extracted DNA at 1000ppm humic acid. Our tests indicate the use of kits that target removal of phenolic products to improve DNA quality may be more important than the use of kits that produce a high quantity of impure DNA. This is especially important with plant tissue that is known to have high phenolic content.

PCR is increasingly being applied in the plant diagnostic laboratory. However, the lack of easy access to positive controls is a major hindrance to incorporation of validated PCR protocols. We described the development and quality testing of cloned plasmid PCR controls to fill this need. By starting with published protocols, we ensured that the amplicons we incorporated into the plasmids were useful and could easily be incorporated into the diagnostic lab process. Additional testing of the protocols and positive controls in five diagnostic laboratories demonstrated the utility of the system

and provided feedback to improve it. Storage and management of the controls could be done in-house or handed over to a culture collection facility focused on that type of service. This system of PCR protocol testing and positive control development and distribution has potential to be used for quality testing of protocols and laboratories.

Table 2-1. DNA extraction kits used by each laboratory during the test of amplification from healthy plant tissue.

Lab	DNA extraction kits
A	QIAmp DNA Stool Mini Kit (cat. 51504, QIAGEN, Germantown, MD) ZR Plant/Seed DNA MiniPrep (cat. D6020, Zymo Research Corp., Irvine, CA) DNeasy Plant Mini Kit (cat. 69104, QIAGEN, Germantown, MD)
B	QIAmp DNA Stool Mini Kit (cat. 51504, QIAGEN, Germantown, MD) ZR Plant/Seed DNA MiniPrep (cat. D6020, Zymo Research Corp., Irvine, CA) DNeasy Plant Mini Kit (cat. 69104, QIAGEN, Germantown, MD)
C	QIAmp DNA Stool Mini Kit (cat. 51504, QIAGEN, Germantown, MD) ZR Plant/Seed DNA MiniPrep (cat. D6020, Zymo Research Corp., Irvine, CA) QBio FastDNA Kit (cat. 116540400, MP Biomedicals, LLC., Solon, OH)
D	QIAmp DNA Stool Mini Kit (cat. 51504, QIAGEN, Germantown, MD) ZR Plant/Seed DNA MiniPrep (cat. D6020, Zymo Research Corp., Irvine, CA) Nucleospin Plant II (cat. 740770.50, Macherey-Nagel, Inc., Bethlehem, PA)
UF	QIAmp DNA Stool Mini Kit (cat. 51504, QIAGEN, Germantown, MD) ZR Plant/Seed DNA MiniPrep (cat. D6020, Zymo Research Corp., Irvine, CA) DNeasy Plant Mini Kit (cat. 69104, QIAGEN, Germantown, MD)

Table 2-2. Thermocyclers used by each laboratory

Lab	Thermocycler
A	Biometra T1(Biometra GmbH, Goettingen, Germany)
B	Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Life Science Research, Hercules, CA)
C	Eppendorf Mastercycler gradient (Eppendorf North America, Hauppauge, NY)
D	Eppendorf Mastercycler gradient (Eppendorf North America, Hauppauge, NY)
UF	Biometra T13 (Biometra GmbH, Goettingen, Germany)

Table 2-3. Description of cloned positive controls

Cloned pathogen target (# amplicons)	Conventional or real-time PCR, amplicon size	Plasmid restriction site(s) containing the amplicon(s)	Antibiotic resistance	Reference for primer pair and PCR protocol
Phytophthora ramorum (2)	Both, 291bp	EcoR1	ampicillin	(Hayden, 2004)
Ca. Liberibacter asiaticus (2)	Both, 317bp and 1160bp	EcoR1	ampicillin	(Jagoueix, 1996)
Raffaelea lauricola (2)	Both, 329bp and 319bp	EcoR1 and ApaI	ampicillin	(Dreaden T. D., 2013)
Clavibacter michiganensis subsp. *michiganensis* (1)	Conventional, 614bp	EcoR1	ampicillin	(Dreier, 1995)
Xylella fastidiosa (1)	Conventional, 733bp		tetracycline	(Minsavage, 1994)

Table 2-4. Cloned pathogen target and plant tissue combinations chosen by each participating laboratory.

	Laboratory				
Pathogen clone, plant tissue	A	B	C	D	UF
Clavibacter michiganensis subsp. *michiganensis*, tomato stems	X		X	X	X
Raffaelea lauricola, swampbay cambium				X	X
Phytophthora sp., azalea leaves	X	X	X		X
Xylella fastidiosa, blueberry midribs	X	X	X		X
HLB (*Ca*. Liberibacter asiaticus), pummelo midribs		X			X

Table 2-5. Sample tube numbers and corresponding calculated concentrations of humic acid. Tube #2 was the negative control (no clone added); 50µL of clone culture were added to all other tubes.

Tube #	Final ppm HA
1	200
2	400
3	356
4	267
5	320
6	133
7	320
8	267
9	356
10	267
11	267
12	200

Table 2-6. Significant effects in the model of lab, lab by kit, host tissue, and kit by host.

Source of effect	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Lab	4	4	16.644774	13.3064	<.0001
Kit	1	1	0.103597	0.3313	0.5663
Lab X Kit	4	4	5.926503	4.7379	0.0016
Host	6	6	16.517994	8.8034	<.0001
Kit X Host	6	6	4.980085	2.6542	0.0201

Table 2-7. Number of sample tubes sent to participating ring-testing laboratories for the first test (healthy plant tissue, extraction with three kits). Each laboratory also ran a non-template control of PCR reagents without plant tissue. Each experiment was repeated once.

PCR target	DNA extraction kit	Number of healthy plant tissue types	Number of replicates	Total samples
ITS	Qiagen Stool	8	3	24
ITS	Zymo Research Plant mini	8	3	24
ITS	1 other, by choice	8	3	24

Table 2-8. Number of tubes sent to participating laboratories, based on the pathogens they chose from Table 2-4. Humic acid concentrations were as in Table 2-5. Each laboratory also ran a non-template control of PCR reagents without plant tissue. Each experiment was repeated once.

PCR target, by pathogen	DNA extraction kit	number of tubes, with varying concentrations of humic acid
P. cinnamomi	Qiagen Stool	12 (tube 2 had no clone)
X. fastidiosa	Qiagen Stool	12 (tube 2 had no clone)
R. lauricola, CHK	Qiagen Stool	12 (tube 2 had no clone)
R. lauricola, IFW	Qiagen Stool	12 (tube 2 had no clone)
Ca. L. asiaticus	Qiagen Stool	12 (tube 2 had no clone)

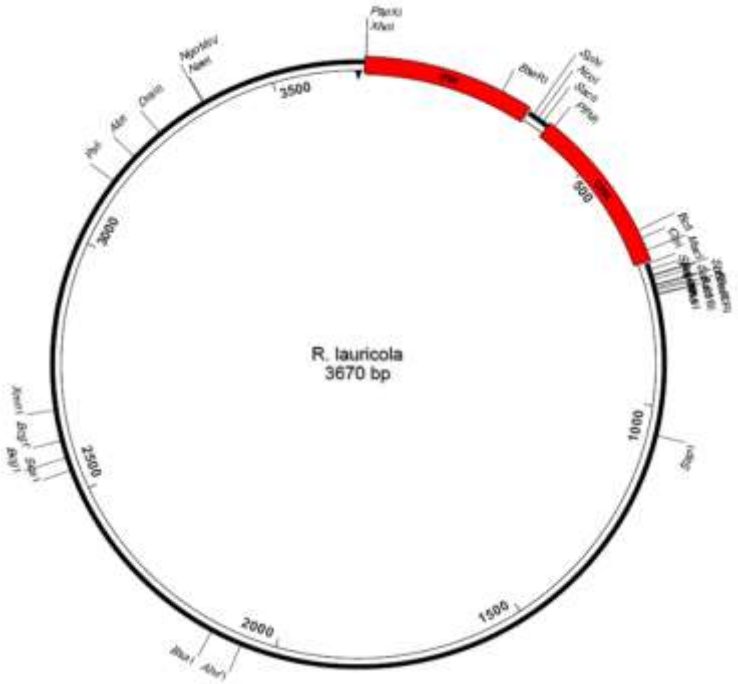

Figure 2-1. Plasmid map of *R.* lauricola targets, “CHK” and “IFW”.

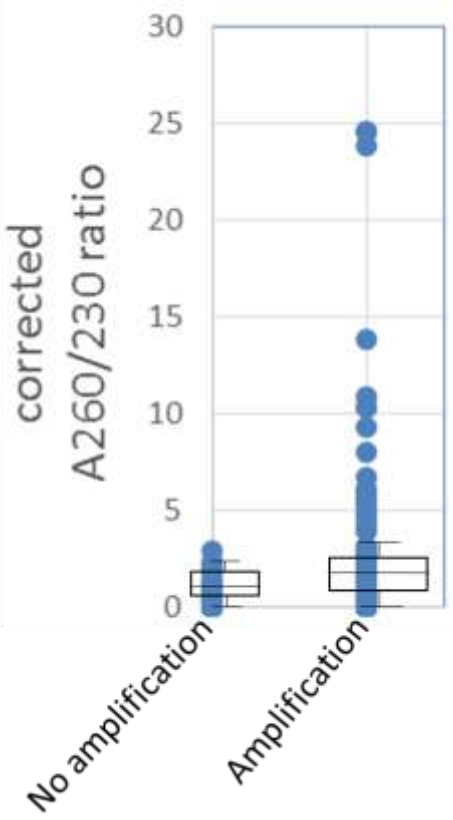

Figure 2-2. Positive correlation between DNA A260/230 ratio >1.6 and amplification for all samples (P=0.0034). The higher the 260/230 ratio, the more likely it was that the DNA would be amplified, especially when the ratio was greater than 1.6. The mean of the A260/230 ratio for all amplified DNA (0.90) was higher than the mean for all non-amplified DNA (0.69). For each set of readings representing DNA that did or did not amplify, bars represent one standard deviation; boxed bars represent the mean.

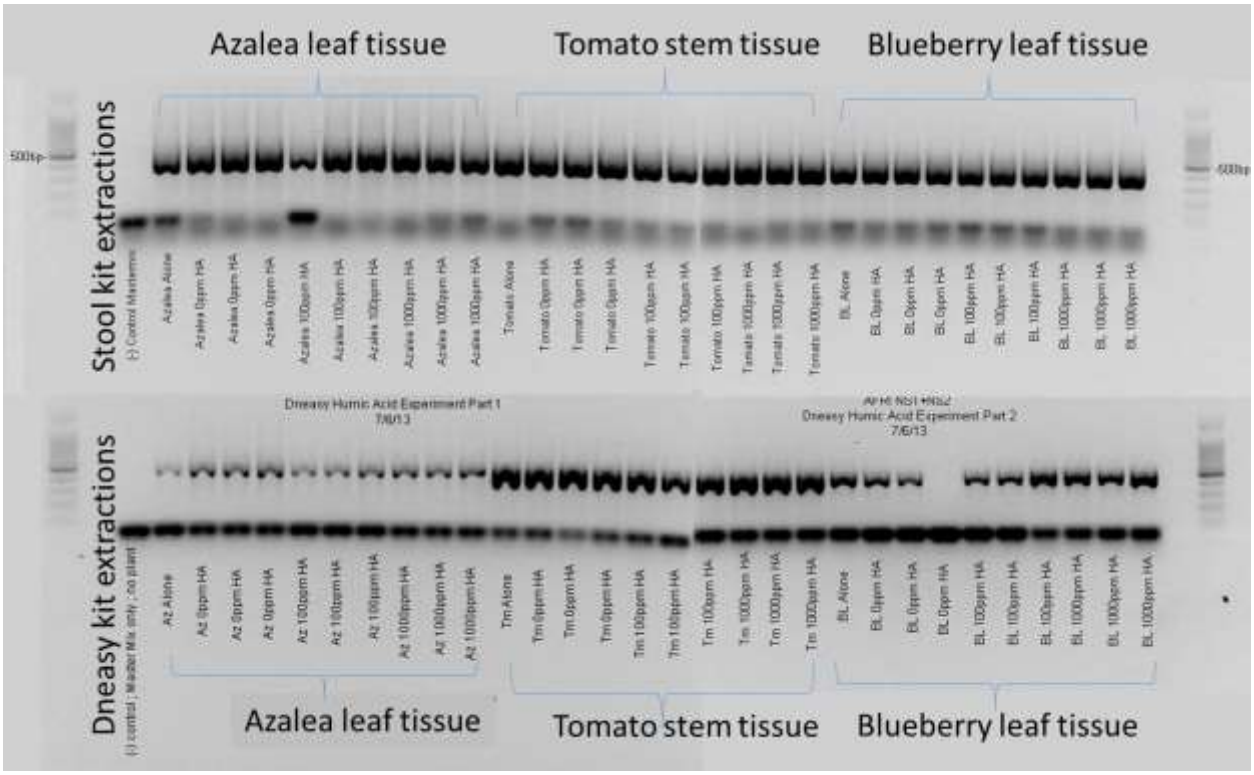

Figure 2-3. Humic acid in concentrations of 0ppm, 100ppm, and 1000ppm does not affect amplification of azalea leaf, blueberry leaf, or tomato stem DNA with NS1-NS2 primers when extracted with Qiagen Stool kit, compared to the Qiagen DNeasy kit, which does not consistently extract amplifiable DNA when HA is combined with high-phenolic tissue such as azalea or blueberry leaf tissue (expected band at 550bp; negative control is water and PCR mastermix).

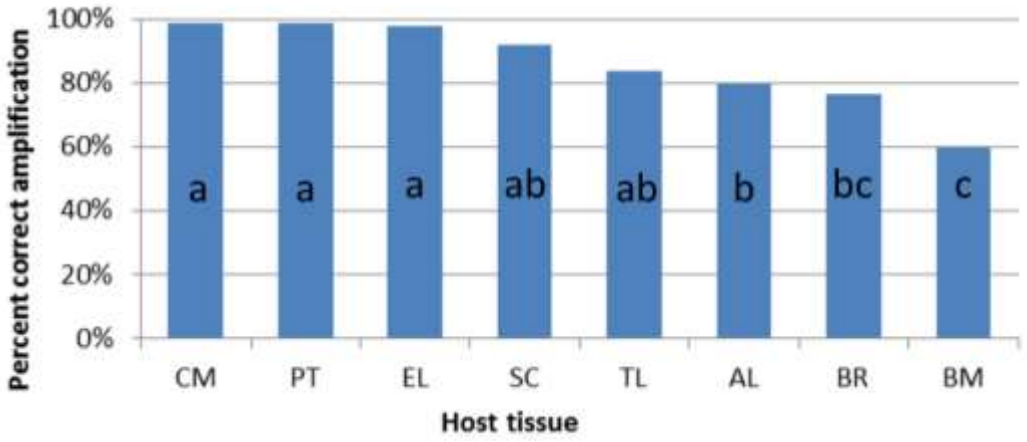

Figure 2-4. Percent of plant tissue samples that produced expected amplicons for all lab-kit combinations. CM=citrus midrib, PT=potato tuber, EL=euphorbia leaf, SC=swampbay cambium, TL=tomato leaf, AL=azalea leaf, BR=blueberry root, BM=blueberry midrib. Columns with the same letter are not significantly different according to LSMEANS with Tukey-Kramer adjustment for multiple comparisons and unbalanced data (α=0.05) following logistic regression in SAS 9.3.

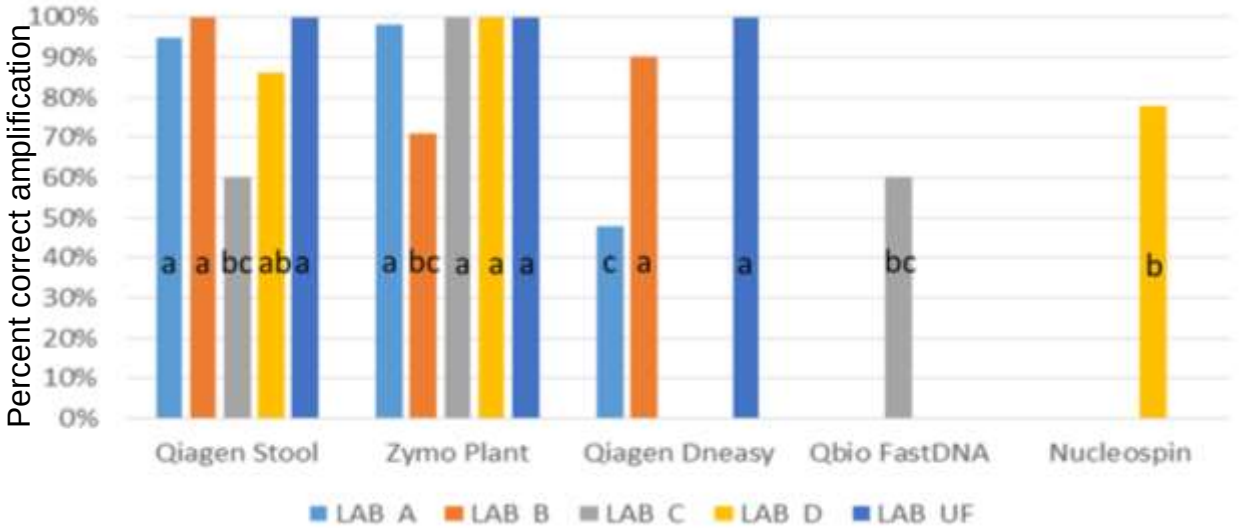

Figure 2-5. Average of amplification by kit across all samples in each of five labs. Across all labs and hosts, the Qiagen stool kit and Zymo plant kit were not significantly different from each other, but were significantly different from the other three kits. Columns with the same letter are not significantly different according to LSMEANS with Tukey-Kramer adjustment for multiple comparisons and unbalanced data (α=0.05) following logistic regression in SAS 9.3.

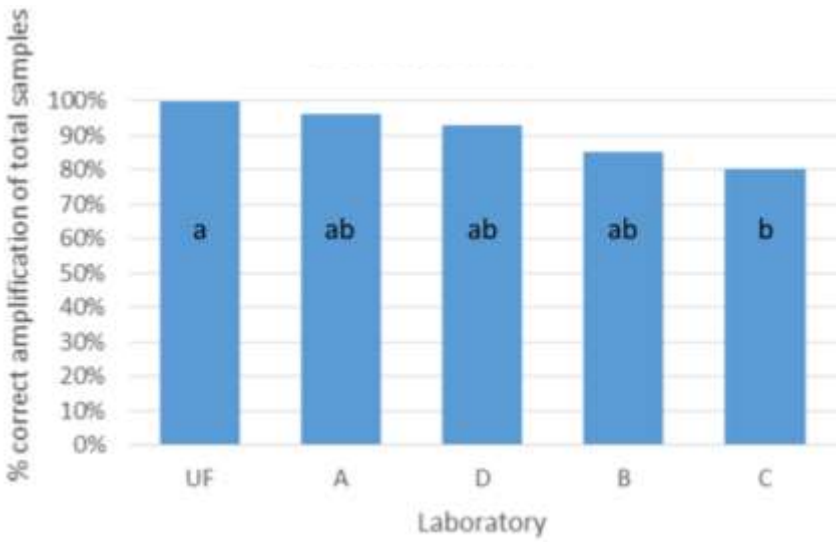

Figure 2-6. Comparison of five laboratories for expected amplification of all samples across all hosts and Qiagen Stool and Zymo Plant kits. Columns with the same letter are not significantly different according to LSMEANS with Tukey-Kramer adjustment for multiple comparisons and unbalanced data (α=0.05) following logistic regression in SAS 9.3.

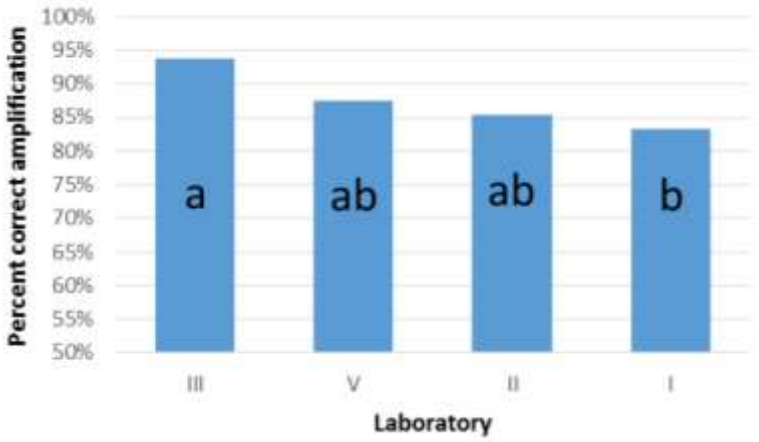

Figure 2-7. Percent of *X. fastidiosa* samples that produced expected amplicons by lab. Columns with the same letter are not significantly different (α=0.05). Significance of pairwise comparisons was calculated using Fisher's exact test with the permutation option and 1000 resamples in SAS 9.3.

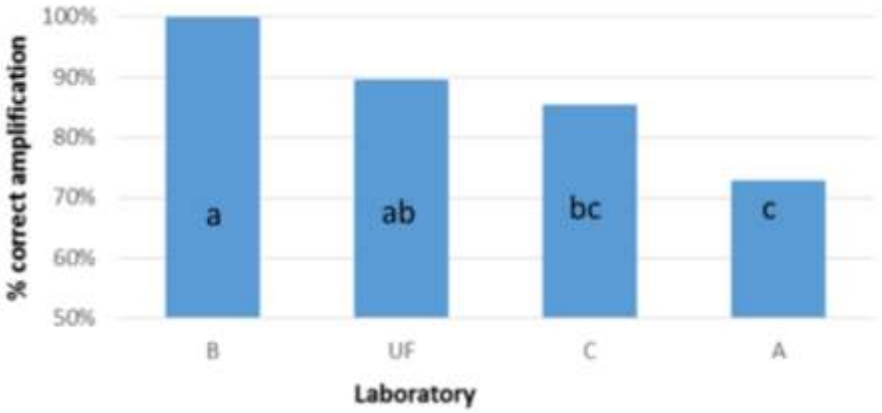

Figure 2-8. Percent of *P. cinnamomi* samples that produced expected amplicons by lab. Columns with the same letter are not significantly different (α=0.05). Significance of pairwise comparisons was calculated using Fisher's exact test with the permutation option and 1000 resamples in SAS 9.3.

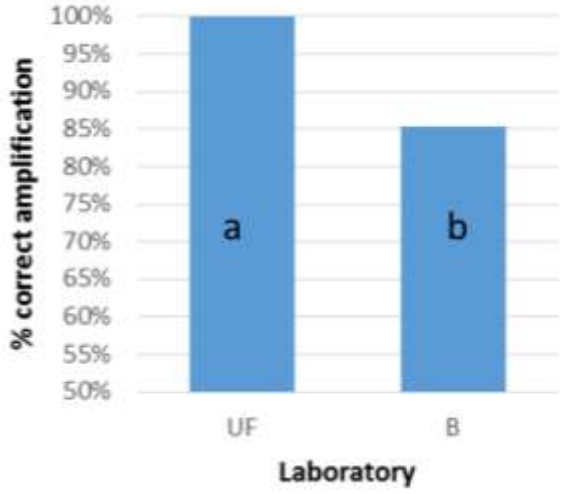

Figure 2-9. Percent of citrus greening samples that produced expected amplicons by lab. Columns with the same letter are not significantly different (α=0.05). Significance of pairwise comparisons was calculated using Fisher's exact test with the permutation option and 1000 resamples in SAS 9.3.

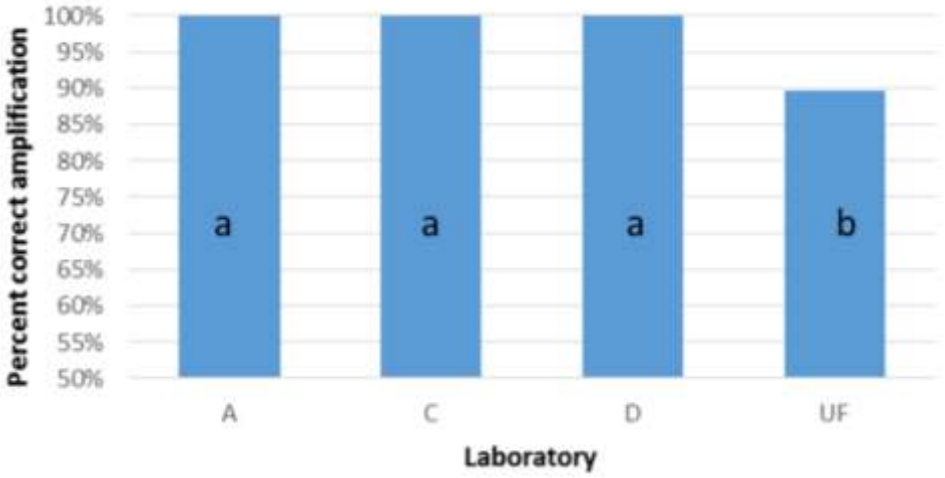

Figure 2-10. Comparison of four laboratories for amplification of the Percent of *C. michiganensis* subsp. *michiganensis* samples that produced expected amplicons by lab. Columns with the same letter are not significantly different (α=0.05). Significance of pairwise comparisons was calculated using Fisher's exact test with the permutation option and 1000 resamples in SAS 9.3.

CHAPTER 3
COMMON SENSE AND SCIENTIFIC VALIDITY IN THE PLANT DIAGNOSTIC LABORATORY: DIAGNOSTIC METHOD DEVELOPMENT POSTULATES AND A MODEL TO SUPPORT RING-TESTING

Background

Plant diagnostic laboratories strive to deliver accurate and timely diagnoses to support effective disease management. Many factors influence the accuracy and speed with which a diagnosis can be rendered, including sampling, submitter information and diagnostic need, condition of the sample, complexity and specificity of chosen tests, diagnostician training and experience, and resources available to support the process. Diagnostic staff must balance these factors against the value of the crop represented by the sample, the cost of the tests, and the level of identification required for management decisions. However, which tests they use, how reliable, accurate, and precise those tests are, and how well diagnosticians perform them, is not standardized for any but the most significant regulatory pathogens.

We propose a system to guide the development of identification assays for diagnostic use, and a risk-based model to evaluate potential reliability considering the validity and robustness of a test and the competence of the lab performing such tests. Initial tests of the model against a test of a protocol in five labs indicated that overall, the protocol was valid under the model parameters set. However, tests of individual labs indicated a significant level of variability by DNA extraction kit, and different kits extracted amplifiable DNA from various host tissues in a significantly different manner. Also, humic acid, reported to confound clean DNA extractions, decreased amplification when combined with plant tissue high in phenolic content, even when the samples were processed with a kit designed to remove phenolic inhibitors. Overall, two kits worked

well for most labs on most host material, and PCR generally worked in most labs most of the time.

Analyses of protocols and laboratories can be standardized to increase lab credibility and diagnostic accuracy and precision, but the expectation of error due to factors internal and external to the testing lab must be considered and defined in a realistic manner. The use of the proposed guidelines and model may assist in the development of accreditation and certification programs for plant diagnostic laboratories.

Articles documenting new or improved identification of plant pathogens are frequently published in peer-reviewed journals. Occasionally, such articles compare multiple methods (Klein, 1997) (Vanysacker, 2010) (Tebbe, 1993) and may even compare the results from multiple laboratories (Jurick, 2007). Diagnostic laboratories are including Polymerase Chain Reaction (PCR) protocols with increasing frequency, but deciding which protocol to adopt between several similar-sounding papers is a daunting prospect. In general, guidance regarding avoidance of errors due to inhibitors, contaminants, etc. is needed before incorporating a new protocol into the roster of common tests in a laboratory. Regrettably, most plant diagnostic laboratories at public universities are minimally staffed and run on shoestring budgets, leaving little time to experimentally compare protocols in the lab or to fine-tune methods that are complicated or don't use off-the-shelf kits or reagents. Most limiting may simply be the nature of the diagnostic service itself; short turnaround time is important if management recommendations are to be useful. This precludes weeks or months of testing multiple protocols, troubleshooting a method, or searching out positive controls.

Diagnosticians meet annually at the national meeting of the American Phytopathological Society and discuss emerging disease issues and the methods used to identify them. This dialog sometimes results in incorporation of new methods recommended by other labs, but rarely results in widespread adoption or even vetting of new methods. Additionally, while there are minutes of the Subcommittee and Roundtable meetings, and electronic copies of relevant posters and sessions, other interactions are not documented and discussion of the robustness or ease of a new method is often lost.

Diagnostic Protocol Resources

The Diagnosticians' Bugwood Wiki Cookbook (http://wiki.bugwood.org/Diagnosticians_cookbook), funded and edited in part by NPDN diagnosticians, is an online, publicly-accessible repository for methods currently in use in plant diagnostic laboratories. These methods have been vetted in multiple laboratories, and the wiki aspect of the site allows for discussion and comments to improve or fine-tune the methods. However, few of the methods currently housed here are molecular tools.

NPPLAP Accreditation

USDA-APHIS-CPHST's National Plant Pathogen Laboratory Accreditation Program (NPPLAP, http://www.aphis.usda.gov/plant_health/cphst/npplap.shtml) and Potentially Actionable Suspect Sample (PASS) program governs non-regulatory and regulatory diagnostic laboratories' diagnostic procedures for a few actionable pathogens. Only those laboratories accredited by the NPPLAP accreditation body and the diagnosticians certified by that body may perform PASS diagnostic tests. Currently, only *Phytophthora ramorum* and the organism that causes citrus greening are covered

by this accreditation. The costs to maintain the specified equipment, supplies, and certification of staff render this program are out of reach for all but a few laboratories in states that generate a large number of these samples. The initial investment alone is more than $35,000 in the specified instrumentation (Cepheid Smartcycler, Cepheid, Sunnyvale, CA) and an estimated $5,000 in reagents and supplies. Additional annual costs include preventative maintenance and calibration contracts of $2,500 or more. Additionally, staff are required to spend approximately a week, and often more than a week, performing the annual certification panel. The cost of this system is borne mostly by the participating laboratory.

NSHS Accreditation

The National Seed Health System (NSHS, http://www.seedhealth.org) administered by the Iowa State University Seed Science Center is another program authorized by APHIS to accredit laboratories. However, the NSHS' scope is limited to accrediting laboratories and other entities to sample or inspect seed and run phytosanitary tests for a specific set of pathogens that restrict international seed movement. The current list of accredited entities is short, and only a small sample of those test for more than a few pathogens. As with the PASS system, the seed test methodologies are available without becoming accredited (Anon. 2013), so any diagnostic laboratory can use them to guide their choice of diagnostic test; however, the results cannot be used for phytosanitary certification. Similarly, members of the European and Mediterranean Plant Protection Organization (EPPO) also publish recommended testing strategies for pathogens of regulatory concern that are accessible without accreditation.

STARD Accreditation

The National Plant Diagnostic Network (NPDN) has developed an audit-based accreditation program that evaluates a laboratory and its activities based on quality assurance through written documentation and periodic internal and external review (Dailey-O'Brien, et al., 2011), to standardize diagnostic methods and reporting. The working group of stakeholder diagnosticians and regulatory authorities are integrally involved in the development and adoption of this accreditation system. Documentation efforts within the accreditation program will assist the laboratories with internal training, recordkeeping, and maintenance and calibration of equipment. However, this System for True, Accurate, and Reliable Diagnostics (STARD) does not recommend specific types of tests or procedures, only that the laboratory has documented procedures and follows its own system of recordkeeping.

US Fish and Wildlife Services Fish Health Centers

Similar systems include food safety and veterinary testing laboratory systems are currently in place to detect causal agents of food-borne illness or animal diseases (University of Arizona Aquaculture Pathology Laboratory, 2005) (True, 2008). These systems have utilized ring tests that include less than ten samples per laboratory. However, the 2008 bacteriological ring-test of the USFWS fish health centers included nine laboratories in the test of their Standard Procedures for Aquatic Animal Health Inspections (SPAAHI, 2007) for detection of four bacterial fish pathogens. Their report indicates that the laboratories did not all use the approved protocols, and their results were not all in agreement. False negatives and incorrect identifications were reported in several laboratories. However, this was a successful ring-test for the laboratories and the protocols in determining limits of detection and laboratory capability.

None of the previously mentioned organizations or systems individually or in combination has a robust system for the development and validation of identification methods for non-regulated pathogens and testing of diagnostic laboratories' capacity to carry out such methods in a repeatable, acceptable, and verifiable manner. We propose just such a system to guide development and validation of new identification methods, and to guide plant diagnostic laboratory staff in grading themselves or others on their ability to carry out such tests.

Objectives

We propose a set of guidelines, describe the limitations and applications of those guidelines, and describe a model for development or evaluation of a method or lab as a starting point for discussion regarding access to and improvement of identification methods in plant disease diagnostic laboratories. Our prime objective is to improve our understanding of risk and competency as those terms pertain to plant disease diagnosis. This system is not meant to be punitive, nor to initiate other action beyond improvement in the way identification methods are developed and the manner in which we determine the validity of results from a given diagnostic laboratory. Additionally, while we focus on the improvement of PCR tests for use in plant disease diagnostic laboratories, the guidelines and model are basic enough to translate to other identification technologies and applications.

Common-sense Guidelines for Diagnostic Method Development

We begin by assuming that the diagnosis should be accurate, timely, relevant, and fiscally appropriate to the submitter's valuation of the crop represented. Additionally, we acknowledge that each of the five guidelines below yields benefits and may have drawbacks.

1. The identification of a pathogen should be based on multiple tests, preferably targeting different aspects of the organism or disease such as metabolism, genome, morphology, symptoms, etc. This is already widely practiced in diagnostic laboratories, but is something that should be taken into account when developing identification tools for new pathogens.

2. A new test should be complementary to existing tests already widely incorporated into diagnostic lab procedures or significantly improve the diagnostic relevance of an existing test either in its accuracy, ease of application in a diagnostic setting, or lower the cost without sacrificing quality of the results.

3. Appropriate positive controls should be developed and a method of dissemination should be addressed. Appropriate negative controls should be described. This will allow for the appropriate use of a test and validation of results.

4. The method should be blind-tested by multiple diagnostic laboratories using appropriate types and numbers of samples. The total number of negative and positive samples should be determined based on the relative risk of a false positive or false negative. A minimum of three labs should be included in the ring test to lower the effect of a single lab.

5. Effort should be made to publish the vetted protocol in a timely manner and to recommend that the publication be made available to diagnosticians via a widely-known resource such as the Bugwood cookbook. This utilizes peer review as the final vetting of the procedure and gets the approved protocol into the hands of those who will need to use it.

Explanations and Limitations

The identification of a pathogen should be based on multiple tests to assist in confirmation of the causal agent of the disease, and to guard against diagnosing incorrectly a saprophyte or other non-pathogen.

Utilization of different aspects of the organism (carbon utilization, coat proteins, pathogenicity genes) and/or techniques (Biolog®, ELISA, MLS) is recommended. A commonly-encountered example of this would be Biolog to assay carbon utilization, coupled with expected PCR amplification and sequencing results. Neither PCR amplification nor Biolog would confirm pathogenicity; diagnosis of the disease requires incorporation of these results with the diagnostician's evaluation of the symptoms, the field information, and the diagnostician's experience with that particular host and/or

disease. In many cases in a diagnostic laboratory, two or more tests coupled with symptoms and the submitted sample information are needed to arrive at a useful diagnosis and resulting management recommendation. However, a modified version of Koch's Postulates (Fredricks, 1996) would be overkill for a relevant and timely diagnosis, unless encountering new pathogens. For some unculturable pathogens such as viruses and phytoplasmas and other fastidious bacteria, only PCR is accessible and easily applied in the diagnostic laboratory if antisera are rare or nonexistent. For example, several NPDN labs currently diagnose Xylella diseases by symptoms and PCR. Diagnosis of asymptomatic tissue is more difficult, false positives and negatives are common, and by these guidelines, would not be recommended for routine diagnosis in plant diagnostic laboratories. A different set of guidelines, such as a certification program like the National Clean Plant Network, should be utilized for this type of sample.

A new test should be complementary to existing tests or significantly improve the diagnostic relevance of an existing test.

The need for multiple tests is one driver of method development, but effort should be made to ensure the new method improves diagnostic confidence and/or is easier to perform than the available tests. A reasonable search of the relevant literature and an evaluation from a diagnostician should discourage the recommendation of overly-complicated or difficult-to-standardize methods, while encouraging methods that target different aspects of the pathogen's biology. Simplified methods that utilize commercial kits and reagents are welcomed by most diagnosticians as they strive to standardize their processes and results across time and laboratories. A streamlined protocol that increases the number of available tests for a particular pathogen is even better.

Appropriate positive controls should be developed and a method of dissemination should be addressed, and appropriate negative controls should be described.

Identification tests are not valid without these controls, and both positive and negative controls are important. Unlike commercially-available ELISA kits that come with positive controls; the widespread availability of standard positive control DNA is a major drawback to the incorporation of PCR in the plant diagnostic laboratory at present. A single research laboratory may have the facilities to store culture collections or infected tissue to provide for positive controls for a limited number of organisms. However, a diagnostic laboratory will not have the facilities to store enough cultures to do so. Unlike a research lab that may perform hundreds or thousands of tests on a single closely-related group of organisms, diagnostic laboratories diagnose a small number of many types of pathogens; storage of many cultures or tissue samples for use perhaps just once in a given year is not usually feasible. It is unlikely that a lab will already have the necessary positive control in hand for every pathogen they diagnose. It should be the responsibility of the method developer to ensure that cloned amplicons or batches of genomic DNA from infected tissue or pure cultures are described and made available if a test is developed for use in identification and diagnosis. Similarly, appropriate negative controls should be recommended in the protocol, even if they are as simple as ensuring the inclusion of a non-template control or DNA from a hole-punch of healthy tissue. It should be noted that most researchers gladly share resources when asked. However, during periods of sample surge or even in preparation for such occurrences, the number of requests for positive control DNA could overwhelm a laboratory. Additionally, the work required to verify the quality of control DNA for a routine number of requests could be impractical for most laboratories. The option of

submitting plasmids to one of several for-profit or not-for-profit facilities set up to provide this service may be a better choice and could further standardize the process. In-house, perhaps a small fee could be charged to cover the cost of creating, storage, shipping, and providing appropriate quality checks.

The method should be blind-tested by multiple diagnostic laboratories using appropriate types and numbers of samples.

Validation of a method for use in a diagnostic lab should be done by diagnosticians. If the validation is performed on multiple platforms, by staff with varying degrees of expertise, and using standard reagents, then the method is also tested for robustness. Not only should a PCR method work for anyone by following the protocol, it should work across multiple PCR platforms, using various commercially-available reagents unless specific reagents are specified in the protocol. Due to the nature of manufacturing, there are differences in products, and it may be necessary to specify reagents such as a polymerase when necessary. Use of kits and familiar reagents increases confidence and standardizes the process across personnel and laboratories. Samples should be homogenized prior to distribution, and extreme care must be taken to ensure the originating lab does not introduce error via contamination of controls, mislabeling, etc. Analysis of the results may assist in tracking such errors if they are made. For instance, a false positive in the same sample across all labs indicates the error was introduced by the originating lab. A standard suite of information should accompany the samples and include the parameters of the test, recommended resources or vendors if applicable, definitions of negative and positive results, and a format for resulting data. A minimum of three laboratories should test the method to reduce the risk of laboratory effect reflecting poorly on the method itself. Our research

has shown that when staff are unfamiliar with a method or even a kit, the error rate increases due to personnel, but not necessarily due to the method itself. When possible, it is recommended that the choice of laboratories be based on their demonstrated capacity to perform similar methods. This serves as a practical, applied peer review of the protocol.

Different risks are associated with false positive results versus false negative results, depending on the risk of not detecting (missing) a true positive or falsely detecting an organism that is not there. Our model is a quick way to determine the minimum number of samples, depending on the number of allowable errors and the confidence in those results. Generally, we would want to be very confident in our assessment of a lab and they would need to have very few errors. The impact of a false positive could mean unnecessary destruction of a crop. Failure to detect a pathogen could allow a pathogen to become established. The impact of wrongly declaring that a lab had failed to correctly detect the true negative and positives could damage the lab's reputation and call into question the value of their service. In short, it is important to blind-test an appropriate number of positive and negative samples, test across multiple appropriate laboratories, and manage the risk of error.

Effort should be made to publish the vetted protocol in a timely manner.

It is important that the protocol find a wide audience in an appropriate peer-reviewed journal, to subsequently be included in a diagnostic resource such as the NPDN Diagnostic Subcommittee website or an open-access resource such as the diagnosticians' Bugwood Wiki Cookbook (http://wiki.bugwood.org/Diagnosticians_cookbook). This allows for a final scientific peer-review of the method, results, and ramifications, as well as opens the door to

future improvements in the protocol and inclusion of helpful information for getting the most out of the method in the laboratory.

Testing the Model

We ran two types of tests of the model. In the first test our goal was to analyze the stringency and practicality of the model, so we set parameters for allowable error and determined the minimum number of samples required to test a protocol across multiple laboratories. Our second test was designed to analyze the capacity of a given laboratory running the same protocol as the first test. Our goal was to determine if we could assign a "grade" to results from an assay performed by staff at a single laboratory, and determine the minimum number of samples required to do so. The data were drawn from the work described in chapter 2 (tests of true positive and negative samples of cloned positive controls and pathogen-appropriate homogenized plant tissue).

Materials and Methods

The Model

The number of acceptable errors is based on the geometric distribution, equation 1, where N is total possible tests, n is the sample size, p is the acceptable error rate, c is the number of allowable errors in sample, and Pa is probability of acceptance. The probability of acceptance is a trade-off between producer risk and consumer risk. As Pa increases consumer risk increases (pronouncing a lab's results good when in reality the results are not good, so pathogens go undetected and cause epidemics, or pathogens are falsely detected and crop destruction or unnecessary sprays ensue). Lower Pa values push the risk on the producer (discrediting a lab's results when they are in reality correctly identifying true positives and true negatives).

$$Pa = P(X \le c) = \sum_{i=0}^{c} \frac{\binom{N*p}{i}\binom{N-N*p}{n-i}}{\binom{N}{n}}, c = 0,1,2,...\min(n, N*p)$$

Based on the hypergeometric equation above and developed in Excel with James Colee, UF/IFAS Department of Statistics, the model is a simple tool to assist in planning a ring test (how many negative and positive samples must be included in the blind test) and grading diagnostic performance of the tests (whether the results are within the allowable error). The equation calculates a geometric distribution of possible tests, confidence, and allowable errors; N*p must be an integer. The dynamic nature of the model allows the user to modify the allowable error to suit the project goals and budget. The model uses hypergeometric acceptance sampling from an infinitely large population (N ≥10000000). An explanation of the terms appears in table 3-1. Example analyses demonstrating the model appear in the results section.

Negatives and positives are defined as true negative and true positive samples, as prepared by an originating lab for analysis at a testing lab. N is a representation of an infinitely large population of possible results and n is the number of possible errors within that population size, based on the setting of allowable error. Allowable error is the percent of total positive or total negative samples that is allowed to be wrong (false negative or false positive). This is a measure of the risk inherent in a false negative or false positive, and is set by the user. An allowable error of 0.01 negatives indicates that for every 100 negative samples analyzed, a lab could have one false positive and would still pass the test. Confidence is a measure of how confident the grader is that the false positive is actually an error on the part of the performing lab, sampling error, or a problem with the protocol. A confidence setting of 0.05 spreads the risk of error across

both the sending and recipient labs, or across the testing agency and the consumer. This also represents the risk to the lab's reputation due to issues outside their control (blind panel samples were mixed up or mislabeled, etc.), while allowable error represents the consumer's risk of a false detection and false alarm or a missed pathogen that might spark an epidemic.

Further Refinement of the Model

Meta-analysis of published data from ring-tests of protocols or laboratory certification systems may be used to test the model. Additionally, virtual data generated by statistical methods such as Monte Carlo simulations could assist in optimization of the model settings and testing the stringency of the model without the cost or technical burden of real laboratory samples.

Results

Application of the Model

Two example scenarios are included here to demonstrate the model when used to determine the number of blind samples to send to a lab. We imagine the utility of this application would be to test the robustness or sensitivity of a new assay. Additionally, the model could help determine the number of samples needed to adequately test a lab's capacity to perform a given test. In both cases, a panel of known positive and negative samples would be prepared by another lab, numbered to blind the samples, and sent to the recipient labs for testing.

Scenario 1:1% allowable error for negatives (false positive results) and 5% allowable error for positives (false negative results).

These might be the parameters to set if a pathogen is endemic, but a closely-related strain is regulated and false detection of the closely-related strain would trigger

crop destruction or heavy losses for the producer (Figure 3-1). The orange and blue lines represent the number of true positive and true negative samples, respectively, required to capture a specific number of errors (false negatives or false positives), based on the parameters set in model for allowable error and confidence.

Scenario 2: 5% allowable error for negatives (false positive results) and 1% error for positives (false negative results).

These might be the parameters to set if missing a detection of a new high-impact pathogen would cause an epidemic or heavy economic impact for the consumer and producer (Figure 3-2). To capture just one error each (one false positive and one false negative), the lab would have to test at least 11 true positive samples and at least 70 true negative samples. Our confidence is represented by the length of the horizontal line at each step. To be very confident in correctly identifying the errors, we should use the numbers of samples indicated by the far-right end of each horizontal line, however, this may represent an unrealistic avoidance of risk; sample numbers indicated by the points along each horizontal line indicate our confidence in the errors that are beyond the lab's control (the blind samples were mixed up or labeled in error by the sending lab, etc.). A more-realistic use of the model might be to spread the risk across both the sending and recipient labs by choosing a sample number somewhere in the middle.

Assessment of the Protocols and Laboratories' Capacity

Table 3-2 contains the data generated by the labs as they conducted the protocols for extraction and amplification of cloned amplicons mixed with healthy plant tissue. The model was used to "grade" the protocols to see if the error was acceptable, based on the above model criteria. The error by lab was also evaluated using the

model to assess the overall capacity of a given lab to process samples via any given protocol.

Assessing the protocols against the model

In our first test, we wanted to assess if the limited number of samples we sent to all testing labs was adequate to assess the risk of error for a given protocol (grading the protocol). Using the error and sample number for positives and negatives and for two given protocols (*C. michiganensis* subsp. *michiganensis* – CMM, and *X. fastidiosa* - XF), we graded the protocols assuming an allowable error of 5% for true negatives (errors = false positives, which could be re-tested to determine if the detection was in error) and 1% for positives (errors = false negatives, which would allow a pathogen to go undetected). We used the minimum number of samples recommended by the model. The scenario under which these allowable error values might be realistic could be propagation trays for tomato transplants (CMM) or blueberry starts (XF), where we want to be sure we have clean propagative material. Any positives could be double-checked to confirm the presence of the pathogen, but if we miss detection of a pathogen, it could spread in the propagative material and in the field.

CMM. According to our model (table 3-3), we should have sent at least 14 true negative samples. We supplied 32 true negatives, so enough samples were sent to adequately assess the negatives, and the error of those samples was 0%. The model indicated we should have supplied at least 70 true positives and we supplied 192, so we sent more than enough to assess the positives, but the actual error was more than twice the allowable error. Using these model parameters, the grades for this protocol would be Fail for false negatives, Pass for the false positives.

XF. The number of supplied samples was identical to the CMM test, so we conclude that we supplied enough true positives and true negatives for a proper assessment (Table 3-4). The false negative error of the processed samples was 9.4%, nearly twice the allowable error of 5%. The false negative allowable error was set at 1%, but the overall error of all the tests for XF was 10.9%. Using these model parameters, the grades for this protocol would be Fail for the false negatives, and Fail for the false positives.

Assessing the laboratories against the model

In our second test, we wanted to see if we sent enough appropriate samples to grade each of the labs on their ability to conduct a specific protocol. Using *X. fastidiosa* as an example again, we again looked at allowable error versus the actual data generated by the labs. Each of four labs received 48 samples, and generated a total error based on giving false positives and negatives equal weight. We set the total allowable error at a moderately-stringent 2.5%, and used the midpoint of the range of samples recommended by the model, representing a higher confidence in our ability to assess the labs without introducing error into the system by mixing up tubes, mislabeling, contaminating the true negatives, etc.. This scenario exists in systems such as the NPPLAPP, whereby diagnosticians are certified via processing of blind panels of samples supplied by an expert laboratory. The expert laboratory has stringent quality checks in place as part of their ISO17025 accreditation.

Lab A. Given a stringent allowance for error (2.5%), the number of samples required for the test (n=45) approximately matched the samples supplied (n=48), so we could assess the lab's performance. The lab's error of 17% results in a failing grade on this test of the protocol (Table 3-5).

Lab B. Given a stringent allowance for error (2.5%), the number of samples required for the test (n=45) approximately matched the samples supplied (n=48), so we could assess the lab's performance. The lab's error of 14% results in a failing grade on this test of the protocol (Table 3-6).

Lab C. Given a stringent allowance for error (2.5%), the number of samples required for the test (n=45) approximately matched the samples supplied (n=48), so we could assess the lab's performance (Table 3-7). The lab's error of 6%, while approaching the allowable, still results in a failing grade on this test of the protocol.

Lab UF. Given a stringent allowance for error (2.5%), the number of samples required for the test (n=45) approximately matched the samples supplied (n=48), so we could assess the lab's performance (Table 3-8). The lab's error of 12.5% results in a failing grade on this test of the protocol.

Summary. Since the protocol received a passing grade, the test of the labs seemed appropriate. A stringent allowable error of 2.5% resulted in a failing grade for all of the labs' results of their testing, even the labs that appeared to do a good job overall. This indicates the model settings may be unrealistic for grading the labs. Further analysis and clearly-defined risk and consequences of error would be required to apply the model in a practical manner.

Discussion

Our tests of the model against scenarios and actual data indicate that it might be useful for designing experiments to test new methods, as well as testing labs in their capacity to perform certain tests. The model we have developed needs further refinement to make it intuitive and to further explore the interaction and interpretation of allowable error and confidence. However, as an exercise, it demonstrates the futility in

developing too-stringent grading schemes for laboratories as well as the utility in defining the number of samples required to address risk and error in a systematic manner. Additionally, analysis of false positives and negatives across labs may help track down error from a common source, such as positive control contamination of negative samples during panel packaging. The true grade of a lab or a protocol would be calibrated by the limits as set in the model, to reflect the inherent difficulty of a type of tissue, for instance, or to reflect the complexity of a protocol or the importance of early detection of a pathogen at low levels. In essence, we can build "handicaps" into the grading to accommodate biological reality. Grades assigned to labs or protocols would need to be accompanied by an explanation of the model parameters and these handicaps to ensure comprehension and relevance. In an ideal world, we would like to have minimal error and be extremely confident in the results. However, in the real world, we have to balance the limitations of laboratories, finances, time, and benefit. Even if this model is not implemented as it stands, it could serve as a starting point for discussion of sampling and accreditation standards that may prove useful in such programs as the APHIS NPPLAP and NPDN STARD.

Useful identification protocols published as the result of research can be more useful to a diagnostic laboratory if a few guidelines are taken into consideration during development and publication of the protocol. Most diagnostic laboratories have molecular tools at their disposal, but need guidance on specific reagents, protocols, troubleshooting, and interpretation of results. Increasing the capacity of a diagnostic lab through improved molecular diagnostics would reap benefits of national and international food, feed, and fiber crop protection. However, there is more to capacity-

building than publishing a set of PCR cycles and accession numbers in GenBank. The goal of this paper is to encourage a thoughtful discussion of recommendations for method development and appropriate testing of the method and even the laboratories themselves. Additionally, we acknowledge financial and other limitations to endless testing that might discourage a scientist from developing a diagnostic protocol and adequately testing it. We believe the middle ground could be defined by modifying our expectations of the rigors of the tests, as demonstrated by the model presented herein. Whether confined by fiscal restraints or defined by economic impact, an appropriate method can be appropriately vetted. The end result is increased diagnostic capacity and increased trust in diagnostic results.

Table 3-1. Parameters of the model. N, n, and confidence are fixed; allowable error is set by the user, based on the risk of false positive or false negative results.

	True negatives	True positives
N	10000000	10000000
n	100000	500000
Allowable error	0.01	0.05
Confidence	0.5	0.5

Table 3-2. Errors in amplification of true positive and true negative samples from a total of 4 sets of 14 samples (12 positive and 2 negative) per lab per pathogen. [1]False negatives were defined as a lack of a visible band on a gel for a sample that should have been positive. [2]False positives were defined as visualization of a band for a sample that should have been negative.

	false negatives[1]	false positives[2]
pathogen (total number samples) # labs	count/total possible, %error	count/total possible, %error
	5/192	0/32
CMM (192) 4 labs	2.6%	0%
	9/96	0/16
HLB (96) 2 labs	9.4%	0%
	19/192	6/32
P. cinnamomi (192) 4 labs	9.8%	18.8%
	1/96	2/16
R. lauricola CHK (96) 2 labs	1.0%	12.5%
	1/96	4/16
R. lauricola IFW (96) 2 labs	1.0%	25%
	21/192	3/32
X. fastidiosa (192) 4 labs	10.9%	9.4%
Overall error	56/864, 6.4%	15/144, 10.4%

Table 3-3. Test of the *Clavibacter michiganensis* subsp. *michiganensis* assay across all labs, testing model parameters against actual assay results.

Model parameters	True negative	True positive	Actual test (CMM)	False positive	False negative
Allowable error	0.05	0.01	Actual error	0	0.026
# samples required	14	70	# samples supplied	32	192

Table 3-4. Test of the *Xylella fastidiosa* assay across all labs, testing model parameters against actual assay results.

Model parameters	True negative	True positive	Actual test (XF)	False positive	False negative
Allowable error	0.05	0.01	Actual error	0.094	0.109
# samples required	14	70	# samples supplied	32	192

Table 3-5. Testing the model parameters against the individual lab results for the *Xylella fastidiosa* protocol and Lab A.

Set 1 Model parameters		Actual test (Lab A)	
Total allowable error	0.025	Total error	0.17
# samples required	45	# samples processed	48
		Pass/Fail/NPD	Fail

Table 3-6. Testing the model parameters against the individual lab results for the *Xylella fastidiosa* protocol and Lab B.

Set 1 Model parameters		Actual test (Lab B)	
Total allowable error	0.025	Total actual error	0.14
# samples required	45	# samples processed	48
		Pass/Fail/NPD	Fail

Table 3-7. Testing the model parameters against the individual lab results for the *Xylella fastidiosa* protocol and Lab C.

Set 1 Model parameters		Actual test (Lab C)	
Total allowable error	0.025	Total actual error	0.06
# samples required	45	# samples processed	48
		Pass/Fail/NPD	Fail

Table 3-8. Testing the model parameters against the individual lab results for the *Xylella fastidiosa* protocol and Lab UF.

Set 1 Model parameters		Actual test (Lab UF)	
Total allowable error	0.025	Total actual error	0.125
# samples required	45	# samples processed	48
		Pass/Fail/NPD	Fail

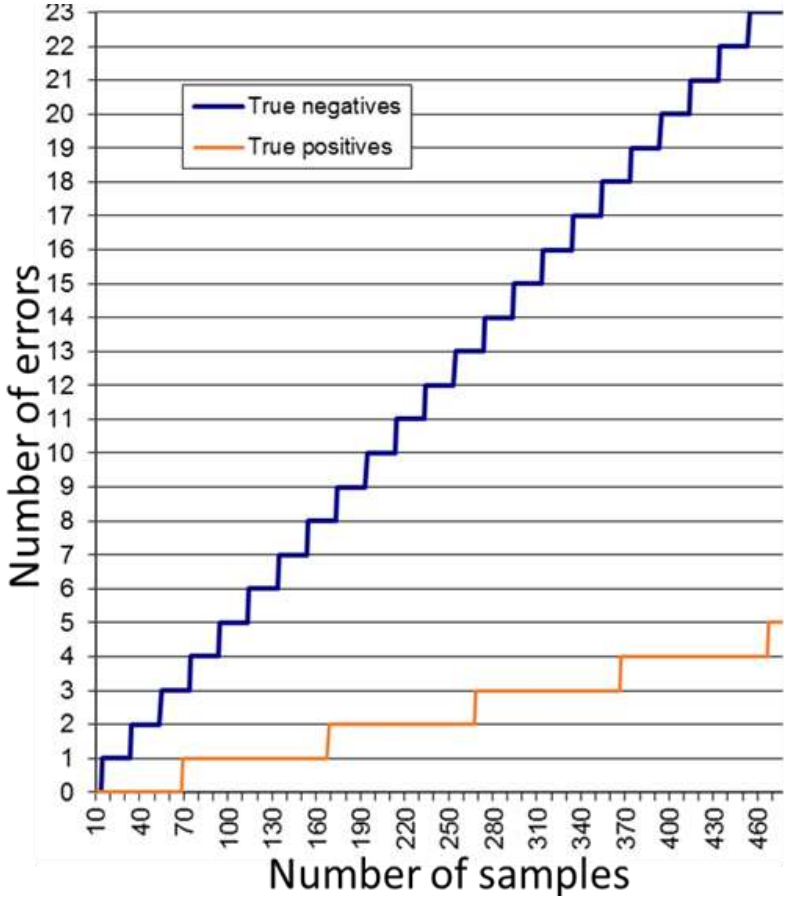

Figure 3-1. Depiction of the model with settings of 1% allowable error for negatives (false positive results) and 5% allowable error for positives (false negative results). The orange and blue lines represent the number of true positive and true negative samples, respectively, required to capture a specific number of errors (false negatives or false positives), based on the parameters set in model for allowable error and confidence.

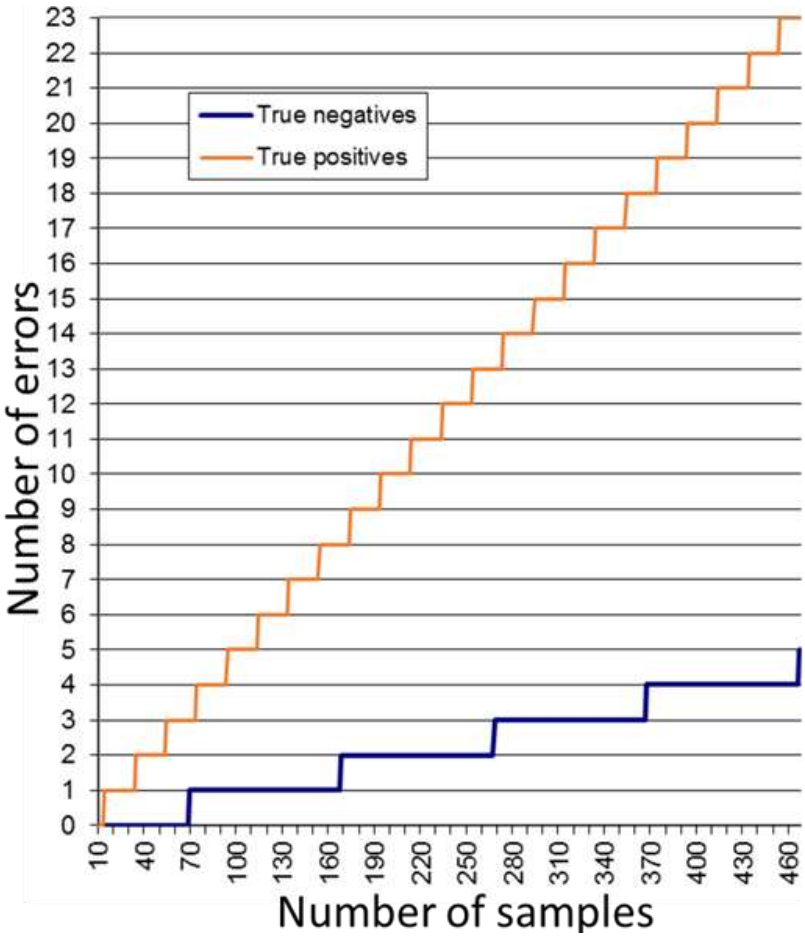

Figure 3-2. Depiction of the model with settings of 5% allowable error for negatives (false positive results) and 1% error for positives (false negative results). The orange and blue lines represent the number of true positive and true negative samples, respectively, required to capture a specific number of errors (false negatives or false positives), based on the parameters set in model for allowable error and confidence.

CHAPTER 4
OLIVE KNOT OR OLIVE NOT: APLICATION OF CONVENTIONAL AND MOLECULAR DIAGNOSTICS TO A NEW CANKEROUS GALL DISEASE OF *LOROPETALUM CHINENSIS* IN FLORIDA

Background

Samples of loropetalum (*Loropetalum chinensis)* with cankerous stem galls were sent from an Alabama nursery to the UF Plant Diagnostic Center in March 2012. Mites and mite feeding injury were noted, and a fluorescing bacterium was isolated from multiple galls. Fall 2012 FDACS-DPI isolated a fluorescing Pseudomonad from similarly symptomatic loropetalums from multiple Florida nurseries. *P. savastanoi* pv. *savastanoi* causes olive knot disease in most olive-producing countries. There is some debate regarding the naming of *Pseudomonas* species and the subspecies/pathovars, including the olive strain of the *Pseudomonas syringae/savastanoi* bacterium (Gardan, 1992) (Hwang, 2005) (Penyalver, 2000) (Ramos, 2012) (Sarkar, 2004). A similar loropetalum pathogen in Alabama in 2012 was identified as *Pseudomonas savastanoi* with a 98% similarity to the 16S sequence of the *P. syringae* pv. *savastanoi* type strain (GenBank accession number AB021402) (Connor, 2013). Florida has a small but growing olive industry, so the presence of the olive knot pathogen would cause concern. Additionally, the rapid spread of the disease within the nurseries represented by samples indicates a need for identification and appropriate management recommendations. Early and accurate detection of the disease is crucial because management of olive knot is dependent on sanitation and cultural practices to eliminate the introduction and movement of the pathogen. A team project between the University of Florida, Department of Plant Pathology and the Florida Department of Agriculture and

Consumer Services, Division of Plant Industry (FDACS-DPI) was developed to diagnose the loropetalum disease.

Initial sequencing of partial 16S ribosomal subunits of the Florida isolates indicated the pathogen might be similar to *P. savastanoi* pv. *savastanoi* and several other fluorescent pseudomonads. Given the importance of the disease in olive-producing areas, and the debate regarding the species synonyms and pathovars of *Pseudomonas savastanoi* (Gardan, 1992) (Sarkar, 2004) (Ramos, 2012), we investigated the identity of the loropetalum isolates with Biolog, fatty acid analysis, LOPAT, multi-locus sequencing, and inoculation studies.

Materials and Methods

Bacterial Strains

Six reference strains, designated LMG 2184, LMG 2209, LMG 2245, LMG 2220, LMG 5385, and LMG 13184, were obtained from BCCM/LMG (Belgian Coordinated Collection of Microorganisms/Laboratory for Microbiology, Ghent University, Belgium). Thirty-two strains isolated from loropetalum, designated13-198 – 13-229, were obtained from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry. One strain (12-254) was isolated from a routine diagnostic sample submitted to the UF/IFAS Plant Diagnostic Center from a nursery in Alabama. One strain (13-474) was provided by K. Connor (Plant Disease Clinic, Auburn University, Auburn, AL), to represent the strains studied in 2012 (Connor, 2013).

Biochemical Tests

Biochemical techniques, Biolog (GEN III MicroLog M System, Biolog, Hayward, CA), fatty acid analysis (Sherlock version 6.1, TSBA6 version 6.10, MIDI Inc., Newark, DE), and LOPAT (Schaad, Jones, & Chun, 2001) tests were conducted on

representatives of 32 isolates from loropetalum samples from Florida, plus an isolate from a loropetalum sample submitted to the UF PDC in 2012 (strain 12-254), an isolate representing the 2013 Alabama *P. savastanoi* publication (strain 13-474), and six reference strains from the BCCM/LMG collection (LMG 2184, 2209, 2245, 2220, 5385, and 13184).

LOPAT. All strains were analyzed for levan production, oxidase reaction, potato rot, arginine dihydrolase activity, and hypersensitive response on tobacco.

Biolog. All strains were analyzed with Biolog 96-well plates using a plate reader (Emax Endpoint microplate reader, Molecular Devices LLC, Sunnyvale, CA) and the Biolog Microbial ID GenIII Microlog plates, software package, and library (Biolog, Haward, CA).

Fatty Acid Analysis. All strains were analyzed with a MIDI Sherlock Microbial Identification System and dendrograms were developed with the strains and several library references (Sherlock version 6.1, TSBA6 database version 6.10, MIDI Labs, Inc., Newark, DE).

Fluorescence. All strains were tested for fluorescence on King's medium B (KMB) (Schaad, Jones, & Chun, 2001). Each strain was streaked onto a KMB agar plate and incubated at 27C for up to 72 hours. Fluorescence was tested by exposing the plate to UV.

Pathogenicity Tests

Stem inoculations of the Florida strains (G13-198 – G13-229) onto loropetalum, mandevilla *(Mandevilla sanderi*), oleander (*Nerium oleander*), and olive (*Olea europaea*) were conducted by syringe injection of bacterial suspensions adjusted to 1×10^8 CFU/ml or inserting a sterile toothpick dipped in a 24 h culture from an NA plate into

young stems. Plants were maintained in a greenhouse at the University of Florida. Inoculations of the two Alabama strains (G12-254 and G13-474) and the six LMG reference strains (LMG 2184, 2209, 2245, 2220, 5385, and 13184) were conducted on loropetalum, oleander, and olive in a containment greenhouse at FDACS-DPI, Gainesville, FL. Each isolate was inoculated at least five times on a stem of each host type. Syringes of sterile tap water and sterile toothpicks dipped in water served as negative controls when inoculated onto each of the host plant types. Plants were checked weekly for symptom development.

Hypersensitive Reaction

All strains were inoculated by infusion of $1x10^8$ CFU suspension into 'Hicks' tobacco leaves and 'Bonnie Best' tomato leaves. Inoculations were checked daily for necrosis.

Preparation of DNA, PCR, and Sequencing

DNA was extracted using the Qiagen DNeasy plant mini kit (cat. 69104, Qiagen, Germantown, MD). Loci chosen for PCR and sequencing included 16S, and iaaL. PCR protocols were performed as published previously (Penyalver, 2000). PCR was performed on all 39 strains with the 16S (FGP) and IAA (iaaL) primer sets. The amplicons were assessed for appropriate size by visualization on a 1% agarose gel, and then were sequenced (Sanger sequencing, ICBR, University of Florida, Gainesville, FL). Sequences were aligned with ClustalW and then analyzed with NCBI BLAST and MEGA 5.2.2 for identity and phylogenetic relatedness.

Positive Control Clone Development

Based on previous success with cloned amplicon positive controls, a portion of the IAA-lysine gene was amplified from the LMG *P. savastanoi* pv. *savastanoi* type

strain (LMG 2209) and cloned into pGEM®-T Easy Vector System I to create a positive control for subsequent PCR.

Results

LOPAT differentiation of the Florida strains (G13-198 – G13-229) resulted in negative reactions for levan production, oxidase production, potato rot, and arginine dihydrolase tests and produced a positive result for tobacco hypersensitivity (Table 4-2). These strains all fluoresced on KMB. Isolates on nutrient agar were creamy in color, and produced smooth, round colonies. Cultures from the LMG collection reacted as above except the strain from trema (LMG 22121), which did not produce an HR on tobacco or tomato, and the chinaberry (LMG 2220), oleander (LMG 5385), and trema (LMG 22121) strains did not fluoresce on KMB (Table 4-2).

Amplification with the IAA-lysine primers (Figure 4-3) resulted in similar bands for strain 12-254, the Florida strains, and the *P. savastanoi* pv*. savastanoi* type strain (LMG 2209), although the olive type strain amplicon appeared to be smaller than the ones produced by the loropetalum strains from Florida and strain 12-254. PCR of strain 13-474 produced a different amplification pattern. Amplification with the 16S primers (FGP, expected amplicon size 1.542 kb) (Figure 4-4) resulted in similar amplification patterns from the two Alabama isolates (12-254 and 13-474), the Florida strains, and the LMG strains from almond (LMG 13184), chinaberry (LMG 2220), loquat (LMG 2184), and olive (LMG 2209). However, BLASTn (NCBI, National Library of Medicine, Bethesda, Maryland) analysis of partial sequences (~900bp) of the ITS region forward from the 16S ribosomal unit (Figure 4-5) and reverse from the 23S of strain 12-254 did not result in a 100% match with any *P. syringae* or *P. savastanoi* accessions*.* PCR amplification of the IAA amplicons from the Florida strains, strain 12-254, and the *P. savastanoi* strains

(3463/3464) from the Jones culture collection indicated that the Florida strains and strain 12-254 were nearly identical. Those strains were very different from the Jones *P. savastanoi* strains 3463/3464 and the amplicons were larger than the LMG olive and loquat strain amplicons, but smaller than the LMG chinaberry and oleander strain amplicons.

We developed a positive control clone of the IAA amplicon. Sequence analysis indicated it is the correct amplicon, and PCR amplification (Figure 4-3) indicates it will be useful as a positive control in future PCR detection and identification of *P. savastanoi* and related pathogens.

Fatty acid analysis (Figure 4-2) of the Florida strains, the Alabama strains, and the LMG strains indicates the Florida isolates are nearly identical to each other and to strain 12-254, but are more similar to the olive (LMG 2209), loquat (LMG 2184), and chinaberry (LMG 2220) pathovars of *P. savastanoi* than to the oleander (LMG 5385) pathovar.

Biolog analysis (Table 4-1) indicated several of the Florida isolates and strain 12-254 were pathovars of *P. savastanoi*. The Biolog GenIII library does not currently contain the olive pathovar. LMG 2209 (*P. savastanoi* pv *savastanoi*, type strain, olive) was identified as *P. savastanoi* pv. *nerii* (Similarity=0.423). LMG 5385 (*P. savastanoi* pv *savastanoi*, oleander) also was identified as *P. savastanoi* pv. *nerii* (Similarity=0.722). The Florida strains were split between mostly *P. savastanoi* pvs. *nerii* and *fraxini*, with the highest probability *nerii*. (Similarity=0.71). Strain 12-254 was also identified as *P. savastanoi* pv. *nerii* (Similarity=0.627).

Inoculation studies of the Florida isolates resulted in cankerous galls (Fig. 4-1) on loropetalum only (Table 4-3). No cankerous galls were observed on wounds from the water inoculations, nor inoculations of the Florida strains onto olive, oleander, or mandevilla. Inoculations of strains 12-254 and 13-474 resulted in cankerous galls on loropetalum only (Table 4-3). Inoculations of the LMG type strains for chinaberry, loquat, oleander, olive, and trema did not result in cankerous galls on loropetalum. Further inoculations of the LMG type strains back to their hosts is ongoing.

Discussion

The evidence from conventional LOPAT, fatty acid, and Biolog analyses indicates the Florida and 12-254 strains could be either the oleander or olive pathovars of *P. savastanoi*. Inoculation studies do not indicate that the Florida strains or strain 12-254 are pathogens of mandevilla, olive or oleander, but the loropetalum-derived strains are all pathogenic on loropetalum. PCR and sequencing of ribosomal and ITS targets indicate the Florida isolates are similar to strain 12-254 from loropetalum but are different from the strain provided by Connor (strain 13-474) (Connor, 2013) and the LMG type strain (2209) of *P. savastanoi* pv. *savastanoi*. PCR of the IAA target indicates the Florida isolates are different from the published Alabama strain (strain 13-474) and are also different from *P. savastanoi* pv. *savastanoi* (LMG 2209). Further sequencing of the rest of the type strains and four other loci is will be necessary to clarify the relationships between the *P. savastan*oi pv. *savastanoi* type strain (LMG 2209), the Florida strains, strain 12-254, strain 13-474, and the Jones *P. savastanoi* strains 3463/3464, but it appears that the loropetalum strain is different from all strains in the *P. savastanoi* group, and could be different from any other strains in the *Pseudomonas syringae* group. Further differentiation with multi-locus sequencing (Maiden, 2006) as in

(Hwang, 2005) may clarify the identity of this pathogen of loropetalum, which will be useful in determining effective management strategies for the ornamental nursery industry in Florida.

Table 4-1. Strain designations, hosts, species/pathovars, and results of Biolog and fatty acid analysis tests.

Strain	host	Location/species	Fatty acid analysis ID	Biolog ID	Biolog SIM
G12-254A	loropetalum	AL nursery	P. syringae and morsprunorum	P. savastanoi pv nerii	0.627
G12-254B	loropetalum	AL nursery	P. syringae morsprunorum and syringae	P. savastanoi pv nerii	0.182
G13-198	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. syringae pv phaseolicola	0.327
G13-199	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.395
G13-200	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. agarici	0.326
G13-201	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.592
G13-202	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. syringae pv delphini	0.387
G13-203	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.511
G13-204	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.17
G13-205	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.211
G13-206	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.374
G13-207	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. syringae pv delphini	0.587
G13-208	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.71
G13-209	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.561

Table 4-1. Continued

G13-210	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. syringe pv antirrhini	0.229
G13-211	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.145
G13-212	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.224
G13-213	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.424
G13-214	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.22
G13-215	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.544
G13-216	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.42
G13-217	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.612
G13-218	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. syringae pv phaseolicola	0.417
G13-219	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.097
G13-220	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.388
G13-221	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.386
G13-222	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.535
G13-223	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.494
G13-224	loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv nerii	0.239
G13-225	loropetalum	FL nursery	P. syringae syringae and morsprunorum	P. savastanoi pv nerii	0.141

Table 4-1. Continued

G13-226		loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. fluorescens biotype F	0.272
G13-227		loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. savastanoi pv fraxini	0.473
G13-228		loropetalum	FL nursery	P. syringae phaseolicola and morsprunorum	P. savastanoi pv nerii	
G13-229		loropetalum	FL nursery	P. syringae morsprunorum and phaseolicola	P. agarici	0.357
G13-405b		blueberry	FL field-grown	P. cichorii	P. chicorii	0.723
G13-474		loropetalum	AL Diag lab	P. putida and agarici	P. fulva	0.796
G13-670	LMG 2184A	loquat	P. syringae pv. eriobotryae	P. syringae glycinea and phaseolicola	Pseudomonas sp.	-
G13-671	LMG 2184B	loquat	P. syringae pv. eriobotryae	P. syringae glycinea and phaseolicola	P. syringae pv pisi	0.523
G13-672	LMG 2209A	olive - Type Culture	P. savastanoi pv. savastanoi	P. fluorescens and glycinea and phaseolicola	P. savastanoi pv nerii	0.423
G13-673	LMG 2209B	olive - Type Culture	P. savastanoi pv. savastanoi	P. fluorescens and phaseolicola	Pseudomonas	0.348
G13-674	LMG 2245A	bean	P. savastanoi pv. phaseolicola	P. fluorescens and phaseolicola	P. syringae pv phaseolicola	0.037
G13-675	LMG 2245B	bean	P. savastanoi pv. phaseolicola	P. putida and corrugata	P. syringae pv phaseolicola	0.049
G13-693	LMG 2220A	chinaberry	P. meliae/P. amydali	P. fluorescens and phaseolicola		
G13-694	LMG 2220B	chinaberry	P. meliae/P. amydali	P. fluorescens and phaseolicola	No ID; P. aeruginosa	0.206
G13-695	LMG 2220C	chinaberry	P. meliae/P. amydali	P. fluorescens and phaseolicola	No ID; P. aeruginosa	0.241
G13-696	LMG 2220D	chinaberry	P. meliae/P. amydali	P. fluorescens and phaseolicola		
G13-697	LMG 2220E	chinaberry	P. meliae/P. amydali	P. fluorescens and phaseolicola	No ID; P. aeruginosa	0.347

Table 4-1. Continued

G13-698	LMG 5385A	oleander	P. savastanoi pv. savastanoi	P. savastanoi nerium		
G13-699	LMG 5385B	oleander	P. savastanoi pv. savastanoi	P. savastanoi nerium	P. savastanoi pv nerii	0.722
G13-700	LMG 5385C	oleander	P. savastanoi pv. savastanoi	P. savastanoi nerium	P. savastanoi pv nerii	0.794
G13-701	LMG 5385D	oleander	P. savastanoi pv. savastanoi	P. savastanoi nerium	P. savastanoi pv nerii	0.703
G13-702	LMG 5385E	oleander	P. savastanoi pv. savastanoi	P. savastanoi nerium		
G13-703	LMG 22121A	trema	P. tremae		P. stutzeri	0.512
G13-704	LMG 22121B	trema	P. tremae		P. aeruginosa	0.535
G13-719	LMG 13184A	almond	P. amygdali/P. meliae	no match	No ID; Burkholderia cepacia	0.266
G13-720	LMG 13184B	almond	P. amygdali/P. meliae		No ID; P. fluorescens	0.204
G13-721	LMG 13184C	almond	P. amygdali/P. meliae	no match	No ID; P. aeruginosa	0.287
G13-722	LMG 13184D	almond	P. amygdali/P. meliae	no match	Staphylococcus	0.422

Table 4-2. Results of tests for LOPAT and fluorescence on KMB.

Strain	Levan production on SPA	Oxidase	Potato rot	Arginine dihydrolase	HR on tobacco	Fluorescence on KMB
G12-254A	-	-	-	-	+	+
G12-254B	-	-	-	-	+	+
G13-198 to G13-229	-	-	-	-	+	+
G13-405b	-	+	+	-	+	+
G13-410b	+	-	-	-	+	-
G13-474A	-	+	-	+	-	+
G13-474B	+	+		+	-	+
G13-670	-	+	-	-	+	+
G13-671	-	-	-	-	+	+
G13-672	-	-	-	-	+	+
G13-673	-	-	-	-	+	+
G13-674	-	-	-	-	+	+
G13-675		-	-	-	+	+
G13-693	-	-	-	-	+	-
G13-694	-	+	-	-	+	-
G13-695	-	-	-	-	+	-
G13-696	-	-	-	-	+	-
G13-697	-	-	-	-	+	-
G13-698	-	-	-	-	+	-
G13-699	-	-	-	-	+	-
G13-700	-	-	-	-	-	-
G13-701	-	-	-	-	+	-
G13-702	-	-	-	-	+	-
G13-703	+	-	-	-	-	-
G13-704	+	-	-	-	+	-
G13-719	-	-	-	-	-	-

Table 4-2. Continued

G13-720	-	-	+	-	-	-
G13-721	-	-	-	-	-	-
G13-722	-	-	-	-	-	-
3463	-	+	+			
3464	-	-	+	-	+	+

Table 4-3. Results of pathogenicity tests on loropetalum, mandevilla, oleander, and olive

PDC or LMG identifier	Loropetalum	Mandevilla	Oleander	Olive
G12-254 A	+	-	-	-
G13-198 to G13-229	+	-	-	-
G13-405b	-	-	-	-
G13-474A	+	N/A	-	-
LMG 2184	+/-	N/A	-	-
LMG 2209	-	N/A	-	+/-
LMG 2220	-	N/A	-	-
LMG 5385	-	N/A	+	-
LMG 22121	-	N/A	-	-
LMG 13184	-	N/A	-	-

Figure 4-1. Injection inoculations of bacterial suspension of the Florida strains resulted in cankerous galls after 4 weeks. Photo courtesy of Carrie Lapaire Harmon, UF Plant Diagnostic Center.

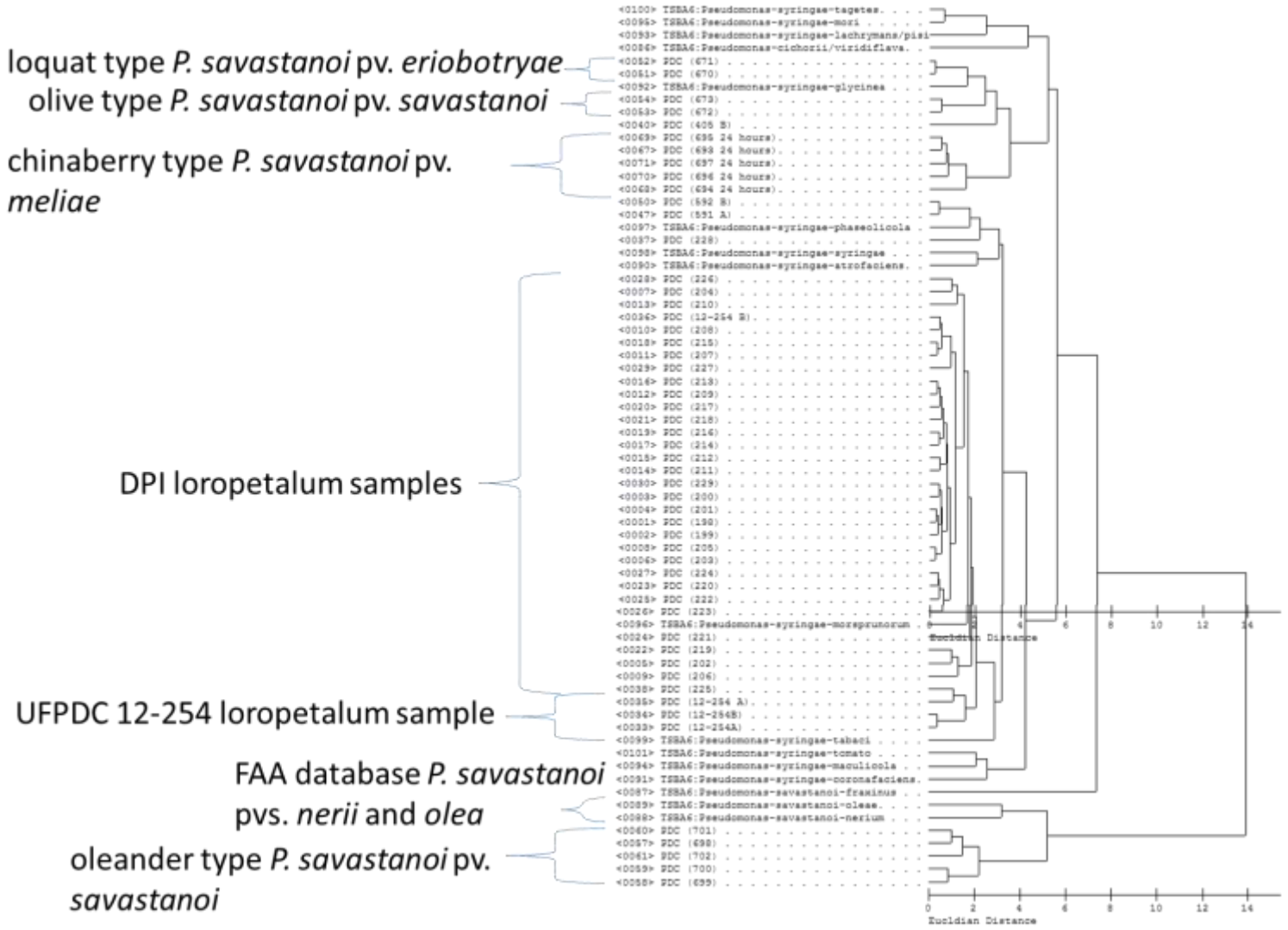

Figure 4-2. Dendrogram of results of fatty acid analysis of LMG type strains, loropetalum strain 254, all Florida loropetalum strains, and Sherlock library references. The Florida strains are identical to each other and nearly identical to the PDC 12-254 strain. The Florida strains are nearly 6 E.D. from the LMG type for olive, and at least 6 E.D. from the LMG oleander strain 5385 and the oleander and olive Biolog library strains.

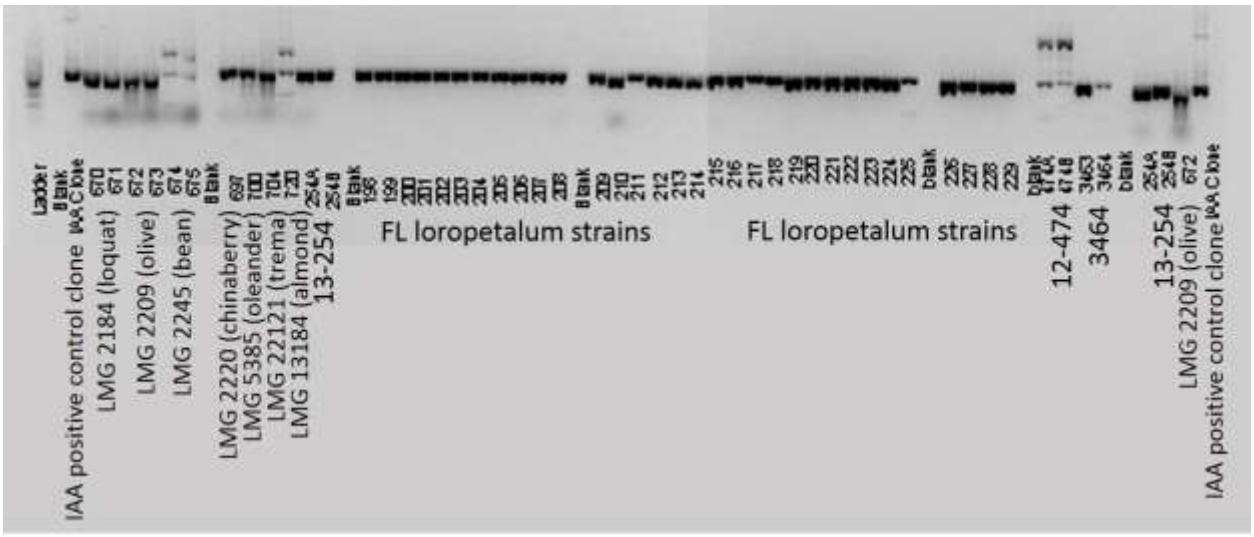

Figure 4-3. PCR amplification with primers for the IAA-lysine synthase gene (*iaaL,* target is 454bp). LMG strains are designated by strain identifier and host; 12-254 is the PDC loropetalum strain from Alabama; 13-474 is loropetalum strains from Alabama published as *P. savastanoi* in 2012; 198-229 are Florida isolates from loropetalum; 3463 and 3464 are strains from the Jones culture collection suspected to be *P. savastanoi*;.

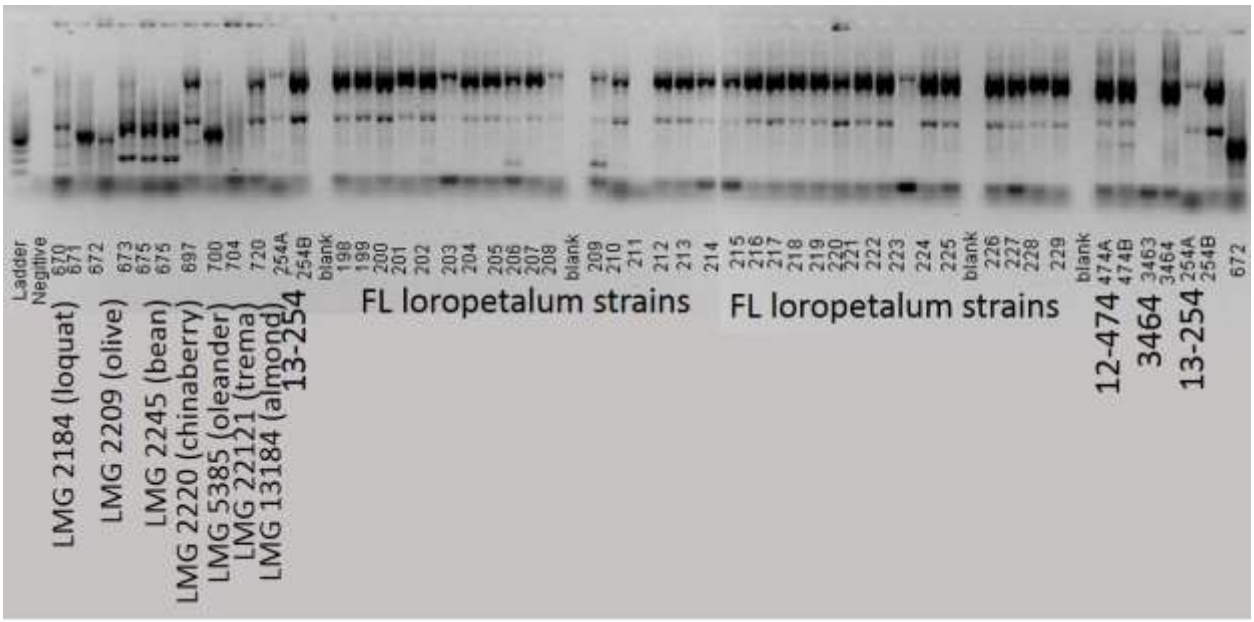

Figure 4-4. PCR amplification with primers for the 16S ribosomal RNA (*FGP,* target is 1.542 kb). The LMG strains are designated by strain ID and host; 12-254 is the PDC loropetalum strain from Alabama; 13-474 is the loropetalum strains from Alabama published as *P. savastanoi* in 2012; 198-229 are Florida isolates from loropetalum; 3463 and 3464 are strains from the Jones culture collection suspected to be *P. savastanoi*.

PSLA-FGPS56

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  1 AGTGCGTGCT GGCAGTCCTA TGACATGCGA GTCGAGCGGA GCCGGGTACT
 51 TGTACCTGGT GGCCAGCGGT GGCGGGTGAG TAATGCCTAG GAATCTGCCT
101 GGTAGTGGGG GATAACGCTC GGAAACGGAC GCTAATACCG CATACGTCCT
151 ACGGGAGAAA GCAGGGGACC TTCGGGCCTT GCGCTTTCTA ATGAGCCTAG
201 GTCGGATTAC CTAGTTGGTG AGGTAATGGC TCACCAAGGC GACGATCCGT
251 AACTGGTCTG AGAGGATGAT CGGCCACACT GGAACTGAGC CACGGCCCAA
301 ACTCCTACGG GAGGCAGCAG GGGGGAATAT TGGACAATGG GCGAAGGGGG
351 GGGCCAGCCA TGCCGCGTGT GTGAAGAAGG TCTTCGGATT GTAAAGCACT
401 TTAAGTTGGG AGGAAGGGCA GTTACCTAAT ACGTGATTGT TTTGACGTTA
451 CCGACAGAAT AAGCACCGGC TAACTCTGTG CCAGCAGCCG CGGTAATACA
501 GAGGGTGCAA GCGTTAATCG GAATTACTGG GCGTAAAGCG CGCGTAGGTG
551 GTTTGTTAAG TTGGATGTGA AATCCCCGGG CTCAACCTGG GAACTGCATC
601 CAAAACTGGC AAGCTAGAGT ATGGTAGAGG GTGGTGGAAT TTCCTGTGTA
651 GCGGTGAAAT GCGTAGATAT AGGAAGGAAC ACCAGTGGCG AAGGCGACCA
701 CCTGGACTGA TACTGACACT GAGGTGCGAA AGCGTGGGGA GCAAACAGGA
751 TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGTCAACT AGCCGTTGGG
801 AGCCTTGAGC TCTTAGTGGC GCAGCTAACG CATTAAGTTG ACCGCCTGGG
851 GAGTACGGCC GCAAGGTTAA AACTCAAATG AATTGACGGG GGCCCGCACA
901 AGCGGTGGA
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Figure 4-5. Partial forward sequence of strain 12-254 from the 16S rDNA into the ITS region.

>PSLA-FGPL132

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  1 TCTGTTTGCC GACTCCCCGA AGCTTTTCGC AGGCTACCAC GTCTTTCATC
 51 GCCTCTGACT GCCAAGGCAT CCACCGTATG CGCTTCTTCA CTTGACCATA
101 TAACCCCAAG CAATCTGGTT ATACTGTGAA GACGACATTC GCCGAAAGTT
151 TGCATTCACA AACTTTACCT TAGCCCGGAC ACGCACCAGT GAAAGAGGTG
201 CCCGGTCTAT TTCTTTCTAT CACATACCCA AATTTTTAAA GAACGATCTA
251 ATCAAAGACT AGAAATCAAC ATTCATGCAC TGATCGTCAG GATCAATGGA
301 ATGCTCATTT CTAAGCTTTC AAACAGAAGC AGTAAGTGGT GGAGCCAAGC
351 GGGATCGAAC CGCTGACCTC CTGCGTGCAA GGCAGGCGCT CTCCCAGCTG
401 AGCTATGGCC CCGTATTTCT ACATACCGTG GAGGCGTCTA CTCTGACCAG
451 CTATCTCACC AGTAAACTGG TGGGTCTGGG CAGATTCGAA CTGCCGACCT
501 CACCCTTATC AGGGGTGCGC TCTAACCAAC TGAGCTACAG ACCCGAACTC
551 GGGCGCTGCT TCTAATCGTC TTCTTCAATG AATCAAGCAA TTCGTGTGGG
601 TGCTTATGGT GCAGCTGAGT CGTCGATTAA GGAGGTGATC CAGCCGCAGG
651 TTCCCCTACG GCTACCTTGT TACGACTTCA CCCCAGTCAT GAATCACACC
701 GTGGTAACCG TCCCCCCGAA
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Figure 4-6. Partial reverse complement sequence of strain 12-254 from the 23S rDNA into the ITS region.

CHAPTER 5
OVERALL SUMMARY AND DISCUSSION

That plant disease diagnosis would be both an art and a science is not obvious at first glance. “Art” is often used to explain the many factors that go into the making of a successful diagnostician: experience, an open mind, application of education, and the ability to take each day as it comes, knowing you will never know it all, and that each day will bring something new. Most plant pathologists with a diagnostic bent add to that the desire to serve a wide and varying clientele and to apply the best science available to their plant disease issues in a way that is timely, practical and affordable. The previous chapters of this dissertation have detailed the science of plant disease diagnosis, from the tools we use today, to the terms we need to more-frequently apply to our assays in the future, such as precision, reliability, and accuracy. This chapter aims to summarize my research that supports molecular tools in the plant diagnostic laboratory, and to imagine what plant disease diagnosis might look like in the future for the Plant Diagnostic Center at the University of Florida.

Detection of plant pathogens is becoming both increasingly important and challenging as global commerce and climate change converge, resulting in the distribution of new hosts and their accompanying pathogens. Early detection and accurate diagnosis encourages appropriate management recommendations and actions ranging from eradication to the use of targeted fungicide spray programs. Diagnosticians frequently encounter challenging samples; organisms may be difficult to culture, difficult to differentiate, difficult to speciate, and difficult to establish pathogenicity. PCR, as one of multiple identification methods utilized, can be applied to increase specificity of identifications, and increase detection of unculturable or

recalcitrant organisms. This dissertation discussed credibility, reliability, accuracy, and precision as they apply to plant disease diagnostics, with specific application of molecular tools.

The research was conducted with the aim to remove some barriers to the use of PCR as a diagnostic tool by recommending guidelines for method development, recommending standard kits that have been shown to reduce PCR inhibitors, and increasing the availability of positive controls. This work proposed a set of guidelines, described the limitations and applications of those guidelines, and described a model for development or evaluation of a method or lab as a starting point for discussion regarding access to and improvement of identification methods in plant disease diagnostic laboratories.

Finally, the work described in this dissertation culminated in application of traditional biochemical and biological diagnostic tests, alongside molecular techniques, to describe what may be a new bacterial pathogen of loropetalum. The work also indicates a limit to the application of universal primers and sequencing in the differentiation of closely-related organisms, especially when basing the comparisons on partial sequences. Diagnosticians will need to be aware of the potential as well as the limitations of molecular tools as we look to these tools to improve routine diagnoses and enable detection of rare or new pathogens.

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