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Genetic Diversity, Prevalence and Incidence of an Invasive Fungus in Puerto Rico: *Coleosporium plumeriae*

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Abstract

Historically, invasive species have been notorious for finding new niches to colonize. Pathogenic fungi are no exception. The rust fungus *Coleosporium plumeriae*, better known as *Plumeria* rust, has invaded *Plumeria* species on a worldwide scale, yet there have been few reports regarding its genetic diversity, and fewer still about its distribution in Puerto Rico. Additionally, the host of interest, *Plumeria alba*, commonly known as white Alelí or Frangipani, has been previously shown to have potential applications for anti-inflammatory colon cancer treatment. Therefore, the conservation of this host may contribute to the development of novel colon cancer medications. The aim of this project is to determine the prevalence, incidence and genetic diversity of the *C. plumeriae* that can be found in different parts of Puerto Rico, as a pathogen on *Plumeria spp*. As with many pathogenic fungi, it is unknown if this fungus reproduces sexually. We hypothesized that if it was introduced to Puerto Rico only once and does not reproduce sexually, the fungus's genetic diversity should be minimal. To determine the prevalence, incidence and genetic diversity, we located *Plumeria* trees, collected infected tissues, extracted DNA, amplified variable genes with PCR, sequenced PCR products and did phylogenetic analysis. We found that *C. plumeriae* is highly prevalent in Puerto Rico and highly related to *C. plumeriae* in Asia. Disease prevalence was also linked to several ecological factors, including elevation, host species and sunlight.

Dedication

I would like to dedicate this work to my wonderful partner Oziel A. Quiles. Thank you for your unconditional love and support throughout this difficult process. You helped me collect samples, reason through the obstacles and hugged me whenever I got frustrated. We both shed tears over this project, and thanks to you I would do it all again in a heartbeat.

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My humblest thanks to the wonderful people at Bayman Lab: Coralis Pagán, Joselyn de Jesús, Ana Medina, Marielys de Jesús, Maylin Caldwell (PhD Student), Naydee Díaz (MSc. Student), Benjamín Mercado (PhD Candidate), Dr. Yobana Mariño, Dr. Luz Serrato, and Dr. Paul Bayman. This work is presented because of our combined efforts to make this happen. Thank you!

A special thank you to José Santana, a retired veteran from Puerto Rico who, after seeing the Facebook post about my search for *Plumeria* trees, kindly dedicated some of his time during the 2020 pandemic to help me locate trees in the northwest region of Puerto Rico. Finally, a heartfelt thank you to my parents- Héctor Ortiz and Lucy Alvarez- for their unconditional love, support and positive energy that allowed me persevere and complete this project. I love you!

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Abbreviations

- ITS- Internal Transcriber Spacer
- PCR- Polymerase Chain Reaction
- DPPH- 2, 2'-diphenyl-2-picrylhydrazyl
- MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
- LCMS/MS- Liquid Chromatography and tandem Mass Spectroscopy
- UH-CTAHR- University of Hawaii-Center for Tropical Agriculture and Human Resources

Section 1: Introduction and Literature Review

Project Overview

This work studies the fungal rust pathogen *Coleosporium plumeriae*, known colloquially as the Plumeria Leaf Rust. Rust fungi are a broad class of pathogenic fungi that exist on a variety of hosts. They are called "rusts" due to the orange color of their spores, which can be found on the back of the leaves they infect. Rust fungi do not grow in culture; therefore, they are difficult to study. *C. plumeriae* is a known pathogen of *Plumeria*, colloquially known as Frangipani or Alelí. Typically used for ornamental purposes, *Plumeria* were recently discovered to have potential biomedical applications in colon cancer drug development, and other anti-inflammatory activities, sparking interest in their preservation (Mistry *et al.*, 2014). However, little is known regarding the genetic diversity, prevalence and incidence of *C. plumeriae* in Puerto Rico; these elements could aid in the preservation of the pathogen's host.

To this end, we set out to locate *Plumeria* trees and determine the infection's prevalence (number of infected trees vs healthy trees) and incidence (number of infected leaves per branch) in Puerto Rico. We hypothesized that the *Plumeria* species in Puerto Rico (*P. alba, P. rubra, P. obtusa*, and *P. púdica*) would be equally susceptible to the *C. plumeriae* infection, considering the island's humid climate, and the fungus's preference for such conditions. Additionally, we hypothesized that *C. plumeriae* would show variation sequence of the Internal Transcriber Spacer (ITS) gene which could point to new information regarding the pathogen's phylogeny. This work, to the best of our knowledge, would be the first of its kind in Puerto Rico.

The specific questions that guided this study are: How prevalent is *C. plumeriae* in Puerto Rico? What percentage of the total leaves are infected (incidence)? What association do ecological factors have with *C. plumeriae* prevalence and incidence? What is the genetic diversity of *C. plumeriae* in Puerto Rico according to its ITS gene? Is there evidence of multiple introductions and/or genetic recombination? Finally, what is the phylogenetic relationship between *C. plumeriae* in Puerto Rico and those found globally?

To answer the first two questions, 151 *Plumeria* trees were located across 16 municipalities and documented their state of infection, as well as several ecological factors (host species, exposure to sunlight, elevation, etc.). The trees were located via convenience sampling,

often by walking or driving within municipalities until more trees were located. A statistical analysis revealed that *C. plumeriae* infection may be significantly linked to host species, elevation and exposure to sunlight, yet not significantly linked to the presence of herbivores (caterpillars), which we believed could have been disease vectors. Spore samples for further genetic analysis were selected randomly. We obtained *C. plumeriae* reference sequences from the global platform GenBank and used them to compare the phylogenetic relationships between this fungus and those found worldwide. This study will add information on the *C. plumeriae* of Puerto Rico, enriching the mycological data on this fungus.

The overall goal of this project was to learn more of *C. plumeriae* from both a distribution and genetic perspective. We also aimed to determine whether there was an association between ecological growth conditions and the fungus incidence and/or prevalence.

Therefore, the paradigm this work follows is a Post-Positivist paradigm. The Post-Positivist paradigm is defined by its pursuit of contextualizing findings and determining patterns or generalizations based on representative samples of a group. This paradigm rejects the idea of a universal truth and attempts to both address and correct the introduction of bias into a sample through both quantitative and qualitative methods (Cooper, 1997). Therefore, this work is consistent with this paradigm due to it being a correlational and mixed study (quantitative and qualitative). It is a correlational study because we were trying to determine whether there exists a relationship between the prevalence and incidence of *C. plumeriae* and diverse ecological factors. Additionally, the study was mixed since the data was obtained from a diverse set of sources, including ITS gene sequences which are qualitative data and disease prevalence and incidence which are quantitative data.

Historical Background of Microbiology and Mycology

Humans have suspected the existence of microorganisms as early as 98 BCE when Roman philosophers began to ponder the existence of smaller lifeforms (Willey *et al.*, 2013). In the 16th century, physician Girolamo Fracastoro was among the first to suggest that disease was caused by invisible living creatures (Willey *et al.*, 2013). However, due to a lack of technology, the existence of microbes remained unconfirmed and a matter of highly-contested conjecture until the late 17th century (Willey *et al.*, 2013). Therefore, it can be inferred that the field of microbiology has been dependent on the advances of technology since its very inception.

The earliest known observations of any organism under a microscope are believed to have been made in 1630 by Francesco Stelluti, an Italian researcher, using a rudimentary microscope probably provided by the prominent scientist and innovator: Galileo. However, Stelluti did not observe microorganisms; he observed microscopically small features on the surface of the bodies of small insects, like bees and weevils (Willey *et al.*, 2013). The first published scientific drawing of a microorganism is attributed to Robert Hooke and his 1665 publication in the journal *Micrographia*. What was the organism he chose to observe and draw? A fungus named *Mucor* (Willey *et al.*, 2013).

Another avid patron and author of the *Micrographia* journal was Antonie van Leeuwenhoek, a Dutch draper and an amateur scientist, who is today regarded as the father of microbiology (Willey *et al.*, 2013). His contributions to science throughout his career were numerous, which surprised his colleagues due to his actual profession being a commercial trader with a notable lack of formal scientific education. In 1674, Leeuwenhoek was the first to design a light microscope that was capable of magnifying an image up to 300 times. This invention allowed him to see beyond the limits of the naked eye and beyond the scope of previous, less powerful microscopes. It was then that he discovered the existence of "small animalcules" or as we would later call them microorganisms, specifically protozoa. He continued to work avidly with his new discoveries and later also observed spermatozoa of dogs and man for the first time in 1677. Additionally, by 1780, Leeuwenhoek observed that yeasts were also comprised of minute particles (Willey *et al.*, 2013).

Around this same time, an Italian botanist was beginning to make some relevant observations of his own. The field of Mycology, a life science field dedicated to the study of fungi, began in the 18th century when Pier Antonio Micheli decided to investigate where exactly fungi come from (Schaechter, 1999). His contemporary, Leeuwenhoek, had already suggested the existence of minute particles in yeasts, which left unanswered significant questions regarding their biological role (Willey *et al.*, 2013). Micheli, continuing this line of inquiry into microorganisms, created a rudimentary experiment that consisted of placing some fungal spores on watermelons and observing what grew (Schaechter, 1999). He observed that the fungal bodies growing on the fruit were identical to the original fungus the spores had come from (Schaechter, 1999). With this, Micheli successfully proved the function of fungal spores. They were the complex, portable,

reproductive structures of fungi (Willey *et al.*, 2013). His work refuted the commonly accepted idea of spontaneous generation that was believed to be the cause of molds and decomposition (Schaechter, 1999). Micheli was the first (and possibly the only person at the time) that was interested solely in the study of fungi (Schaechter, 1999). While Leeuwenhoek did look at some yeasts in his time, he was mostly focused on protozoans and bacteria (Willey *et al.*, 2013). After his watermelon experiment, Pier Antonio Micheli left the field of botany and mostly specialized in microfungi (Schaechter, 1999). He later discovered important genera, such as *Aspergillus*, and became posthumously recognized as the father of modern mycology (Schaechter, 1999).

Hooke, Leeuwenhoek and Micheli each made their own significant contributions to their respective, yet intimately related fields. The early, single lens microscope and close observations of new microscopic species revolutionized the way science was conducted for years to come. From these humble beginnings and thanks to technological progress, mycology has grown from rudimentary observations of fungi in the wild to observing them under microscopes in laboratory settings to currently studying their genomes at the molecular level.

Genomics and Mycological Study

Usually, science has to wait on technology. Mycology is no exception. Like all scientists, mycologists often embrace new technologies that permit them to study biological functions and relationships of fungi, such as the case of the compound light microscope. Though initially developed as early as the 16th century, the compound light microscope as we know it today was not perfected until the late 1860s, when Ernest Abbe and Chester Moore Hall invented new kinds of lenses that could fix the chromatic aberrations that had plagued the earlier compound microscopes (Mansuripur, 2002). For well over a century, their discoveries made this microscope the go-to method for mycologists and microbiologists alike to closely study their organisms (Mansuripur, 2002).

Decades later in the 1960s, a new revolution in the field of microscopy arrived, the electron microscope (Bracker, 1967). This new microscope allowed mycologists to see fungal spores and hyphae at the intracellular level. This revolutionized the way mycologists were able to draw conclusions regarding certain biological functions and happenings within fungal cells. It led to a boom in systems biology publications regarding the inner workings of fungi (Bracker, 1967).

The next big advance for mycology came in the late twentieth century. Gene sequencing began to be developed by Fred Sanger and colleagues in 1965 (Heather & Chain, 2016). They used RNA strains at first because they were easy to manipulate and already known to be rather short in sequence length. RNA sequences were also less complicated due to a lack of complimentary strains polluting the sample (Heather & Chain, 2016). These factors made them ideal test strains to develop and fine tune the gene sequencing technique (Heather & Chain, 2016). Thanks to further advances in technology, and the arduous work of scientists like Sanger, DNA sequencing came into the mix in the late 1970s (Heather & Chain, 2016). In this work, we will be sequencing fungal DNA (Heather & Chain, 2016).

By the end of the 20th century, the development of Polymerase Chain Reactions (PCR) led to a second publication boom in the field of mycology and widely spread the use of phylogenetics in the study of fungi (Hibbett *et al.*, 2013). This important molecular technique will be utilized in this study.

Gene sequencing of fungal DNA led to a better understanding of fungal relationships within and between species. Sequencing fungal DNA brought a new realm of genetic information into play. In this work, to address our questions on genetic diversity and phylogenetic relationships, we will be sequencing the Internal Transcriber Spacer (ITS) gene. This gene is commonly used in the study of fungi because it was shown to have a high specificity for a broad range fungi, and includes variable regions between highly conserved sequences that can be used to develop PCR primers, making it an ideal 'barcode' for fungi identification (Schoch *et al.*, 2012).

Gene sequencing has changed the fields of genetics and mycology forever. As sequencing technology developed, a larger breadth of genetic information was gathered for different fungal species. Though initially a slow-going process, whole-genome fungi publications have been growing at an exponential rate since the beginning of this century (Hibbett *et al.*, 2013). The first fungal genome, sequenced in 1996 by André Goffeau and his team (Goffeau *et al.*, 1996), sparked interest in sequencing fungi species of experimental importance, such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Neurospora crassa*. Initially, the development of gene sequencing was slow because the technique's high cost made it prohibitive (Willey *et al.*, 2013). For example, the Human Genome Project was able to sequence the whole human genome in 2001 at a cost of $300 million dollars (Willey *et al.*, 2013). Thanks to technological advances

in what has been dubbed "next-generation sequencing methods," today you can get a whole human genome sequenced for a couple of thousand dollars (Willey *et al.*, 2013). The ability to quickly and affordably sequence genes has also allowed a new branch of mycology to fully blossom: fungal phylogeny (Hibbett *et al.*, 2013).

Fungal Phylogeny, Evolution and Invasive Species

Every fungal species in the world has its own evolutionary history. Each species has adapted and evolved into the species we see today. Though originally lumped into the Plant Kingdom by Linnaeus (Linné, 1789), we have since learned that fungi are neither animals nor plants, but a different kingdom, Fungi. Fungal phylogeny was historically determined using traditional methods such as fossils and physical morphology (Ahmadjian, 2020). Today, the relationship between all species, as well as between fungal species, becomes more easily apparent through gene sequencing. Thanks to the development of gene sequencing, scientists have been able to identify new fungal phyla that would have otherwise remained undifferentiated from other phyla. The differences between these phyla are sometimes only evident at the genome level, but most are today easily differentiated using single ribosomal genes (Jones *et al.*, 2011). Since fungal phylogeny is now more readily tracked through gene sequencing, it is possible to track where species and even races may have come from by comparing them to other species in the GenBank database. It is through these comparisons that scientists are able to draw conclusions about the relationship between fungal species from different parts of the world, and use that data to reconstruct that fungus's evolutionary history. Before genome sequencing, individual gene sequences were the only way to determine relationships among fungal populations (Choi & Kim, 2017). However, with genome sequencing, scientists use whole-genome analyses to produce "genome trees" for fungal species, offering a more detailed and accurate evolutionary picture (Choi & Kim, 2017). These trees establish a "genealogy" for the fungi being studied by tracking their evolutionary history (Choi & Kim, 2017).

Knowing the evolutionary history of a fungus can help determine if it is native or invasive to the territory where it is found (Mooney & Cleland, 2001). Invasive species can cause significant changes in the environments they inhabit, but their new environments can also favor genetic changes within them (Mooney & Cleland, 2001). Gene sequencing provides scientists with a tool to study invasive species at a genetic level and make inferences about their evolutionary history

(Mooney & Cleland, 2001). This information improves predictions on the behavior of the invasive species which will inform conservation efforts to curb their spread. In my work, genetic sequencing will allow us to phylogenetically relate *C. plumeriae* in Puerto Rico to other *C. plumeriae* worldwide. This may provide us with insight into where our strain may have been introduced from. It will also enrich the genetic data available on this fungus.

Symbiosis Between Organisms

Fungi are commonly found in a variety of relationships with the environment that surrounds them. They can be saprotrophic, mutualistic or parasitic (Palm, 2001). Saprophytic fungi obtain their necessary nutrients by consuming and breaking down dead plant or animal tissues (Palm, 2001). Mutualistic fungi have beneficial relationships with their host, in which both parties gain something from their union (Palm, 2001). A perfect example is mycorrhizal fungi that help the roots it lives on uptake greater amounts of nutrients from the soil without killing them (Palm, 2001). Meanwhile, parasitic fungi live off their host long enough to reproduce and then kill off their host (Palm, 2001). There are some parasitic fungi that are considered obligate parasites because they are unable to grow without a living host (Palm, 2001). These types of fungi include powdery mildews, downy mildews and rusts (Palm, 2001). *C. plumeriae*, a rust, is categorized as an obligate parasite.

Fungi also infect human hosts. Infection can range anywhere from a slight annoyance to a potentially fatal disease. These pathogens have different points of entry into the human body, be it through the skin, open wounds or mucous membranes (Calderone, 2001). Some fungal pathogens are a natural part of the body's microbial flora. They become opportunistic pathogens when they take advantage of immune-compromised hosts (Calderone, 2001). Some examples of opportunistic fungi are *Candida albicans* and *Aspergillus fumigatus*. *Candida albicans* commonly causes *Candidiasis* infections, rashes in the skin, mouth, and genital regions (Calderone, 2001). Meanwhile *Aspergillus fumigatus* is one of the most dangerous airborne pathogens in hematology that infects immunocompromised patients. It is characterized by severe lung infections and is often fatal (Calderone, 2001). Their opportunistism and similarity to human cells complicates potential treatments (Calderone, 2001).

As an obligate parasite, *C. plumeriae* is highly dependent upon its host. To be able to reproduce, grow and spread to other hosts, it requires the stability given by its host plant. This is true for all rusts. Yet, not every rust reproduces in the same manner.

The Rudiments of Fungal Reproduction

Fungi reproduce in several ways. For the purpose of this study, we will focus on the known modes of reproduction within the Order *Pucciniales*, of which *C. plumeriae* is a member. *Pucciniales* comprises thousands of obligate parasite species, better known as rusts, which are found on ferns, conifers and flowering plants (McTaggart *et al.*, 2016). These rusts are devastating parasites on many crops and attack more than 7,000 hosts (Fernandez *et al.*, 2013). The mode of reproduction of the rust of interest in this work is still mostly unknown. The typical life cycle of a rust fungus has been found to be either macrocyclic, microcyclic or demicyclic (Fernandez *et al.*, 2013). A macrocyclic rust is one that undergoes all five stages of reproduction: basidiospores, pycniospores, aeciospores, urediniospores, and teliospores (Fernandez *et al.*, 2013). This type of cycle is shown in **Figure 1**. During the first three spore stages (basidiospores, pycniospores, aeciospores) the rust is able to infect a living plant host through the stomata or any other natural openings. The urediospores are able to re-infect the same host and are the stage most likely to spread the fungus (Fernandez *et al.*, 2013). The airborne aeciospore stage transports to its secondary host, its telial host (Fernandez *et al.*, 2013). A microcyclic rust is one that undergoes only a couple of the aforementioned spore stages, as shown in **Figure 2,** where only basidiospores and teliospores are represented in this rust's life cycle (Aime *et al.*, 2017). Microcyclic fungi almost always lack uredinial and aecial spore stages (Aime *et al.*, 2017).

Furthermore, a demicyclic rust is similar to the macrocyclic, but lacks the repeating uredinial stage (Salama *et al.*, 2012) and can occasionally lack spermogonia and spermatia as well (Petersen, 1974). Both demicyclic and macrocyclic rusts can exist as heteroecious and autoecious species (Petersen, 1974). A heteroecious rust is that which requires two hosts in order to produce all of their reproductive structures and be able to reproduce effectively (Petersen, 1974). Meanwhile, autoecious rusts only require one host (Petersen, 1974). An example of a demicyclic rust cycle can be found in **Figure 3**.

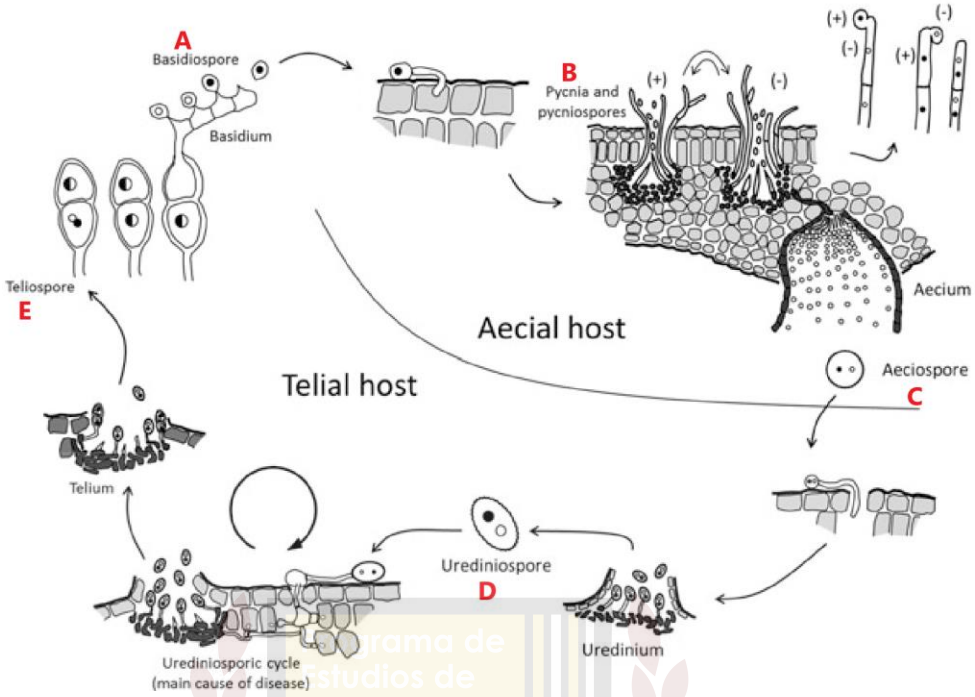


Figure 1*: Macrocyclic Life Cycle of Rust Fungi.* This figure demonstrates all five stages of the life cycle of a typical heteroecious rust fungus. Here we highlight the five relevant spore stages in alphabetical order. A) Basidiospores, B) Pycniospores, C) Aeciospores, D) Urediniospores, and E) Teliospores. The telial host is the primary living plant host whereas the aecial host is the secondary host of its cycle. From (Fernandez *et al.*, 2013).

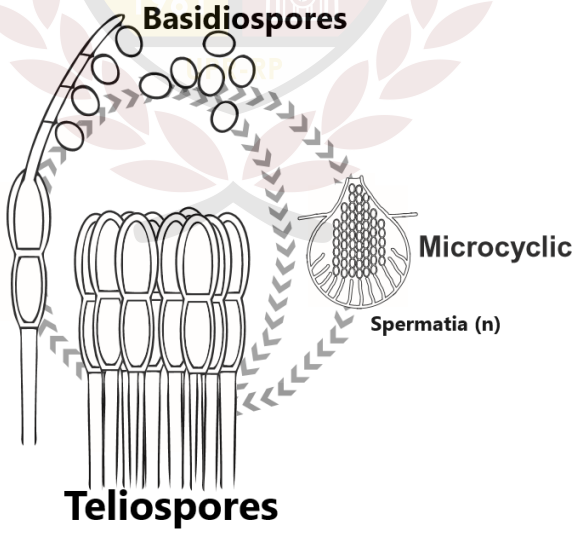


Figure 2*: Stylistic Representation of a Microcyclic Rust Fungus' Life Cycle.* from (Aime *et al.*, 2017)

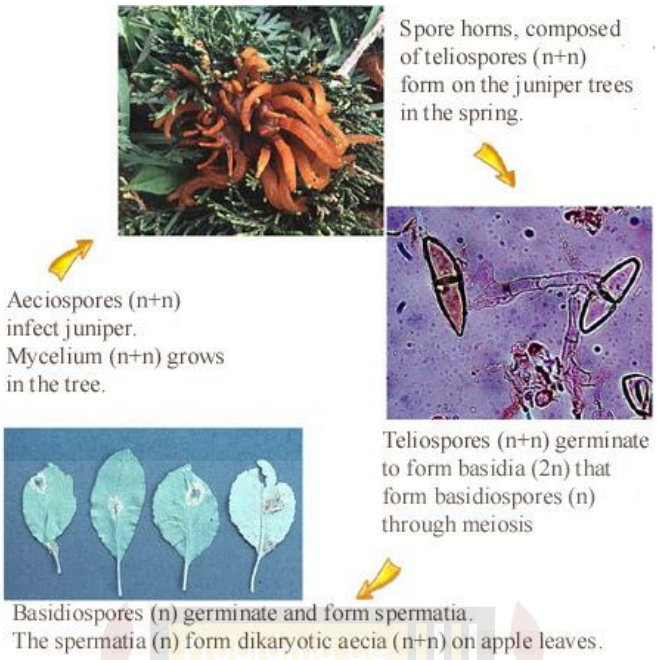


Figure 3: *Life Cycle of Demicyclic Rust Fungus*. This example of a demicyclic rust life cycle belongs to a common apple cedar rust named *Gymnosporangium juniperi-virginianae.* It is important to note the lack of a urediniospore stage, characteristic of a demicyclic rust. Adapted from (Volk, 1999).

We assume the mode of reproduction of *C. plumeriae* is similar to the life cycle for the coffee leaf rust, *Hemeleia vastatrix* because they share spore stages of reproduction. Coffee leaf rust is the most destructive disease that affects coffee in Puerto Rico and around the world. It has caused devastating crop losses in nearly all coffee-producing countries (Agrios, 2005). While it attacks every species of coffee, it is more severe on *Coffea arabica* (Agrios, 2005). *Hemeleia vastatrix* is hemicyclic, which means that its spermagonial and aecial states are currently unknown (Talhinhas *et al.*, 2017). Due to the presence of urediniospores, it is not a demicyclic rust. The reproductive structures and life cycle of the coffee leaf rust can be found in **Figure 4**.

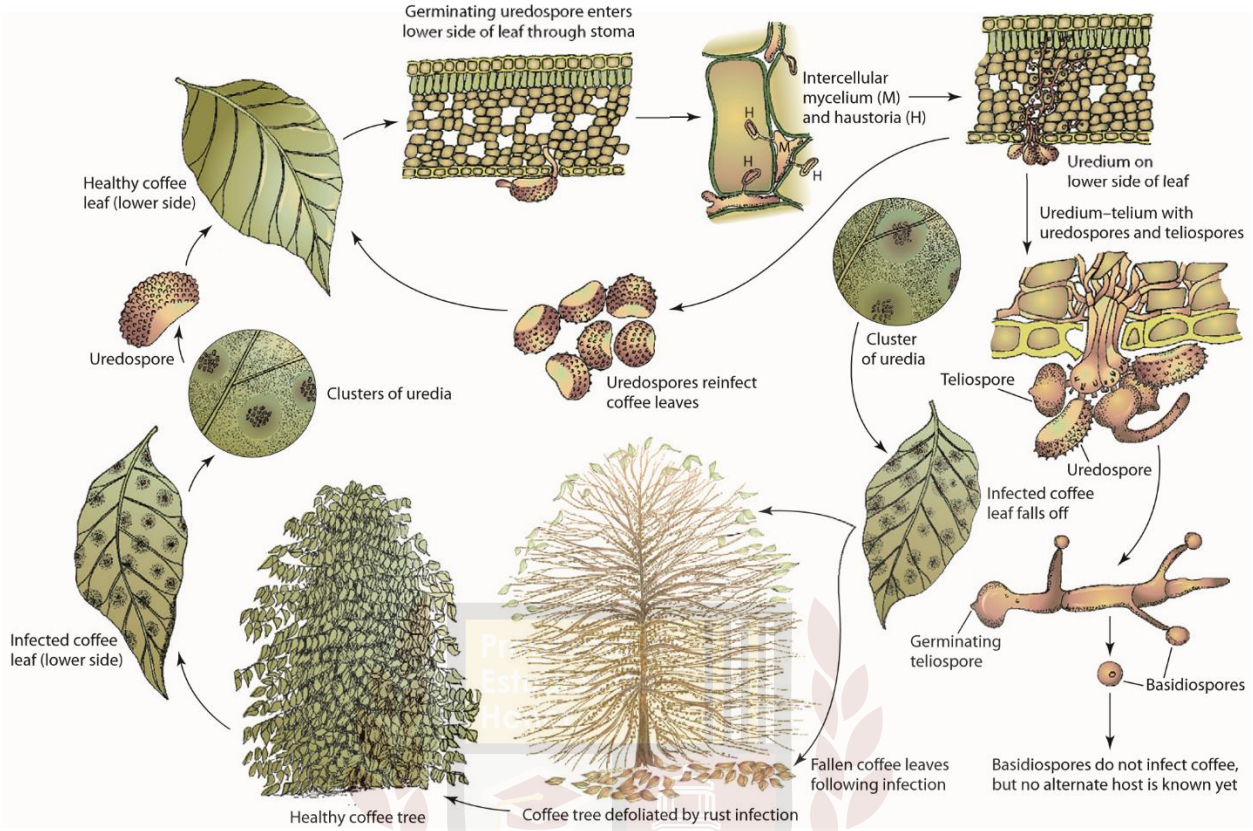


Figure 4*: Coffee Leaf Rust Life Cycle with Reproductive Structures.* The figure demonstrates all three known reproductive stages of *Hemeleia vastatrix*, the coffee leaf rust: basidiospores, urediospores and teliospores. The spermogonia and aecial stages are currently unknown. from (Agrios, 2005)

In the case of *C. plumeriae*, the life cycle is thought to be likely hemicyclic and autoecious with no alternate host because the uredial, basidial and telial stages have been reported, but the spermagonial and aecial stages are still unknown (Chaijuckam *et al.*, 2020; Nelson, 2009). The genetic information gleaned from this work adds to the current knowledge surrounding *C. plumeriae's* mode of reproduction.

Rusts represent a significant threat to human health, food and drug accessibility and security. Rusts are significant pests of important medicinal plants such as mint, marjoram, and wormwoods, as well as to important agricultural crops such as wheat, rice, soybean, sugar cane and coffee (Margina & Zheljazkov; Yamaoka, 2014). In fact, in 2009, invasive fungal pathogens as a whole caused a loss of $21 billion for the agricultural market in the United States alone (Rossman, 2009). These losses and threats to important crops indicate that rust fungi represent a great danger to economic and agricultural stability. Similarly, mint is well-known as a biomedically important crop worldwide which is also affected by a rust (Margina & Zheljazkov,

1994). In Bulgaria, a 1994 report found that the mint rust, *Puccinia menthae*, was the biggest threat to mint production. Over 50% loss of the mint crop each year has been attributed to mint rust (Margina & Zheljazkov, 1994).

Plumeria Rust Disease in Puerto Rico

The most recognizable symptom of Plumeria Rust disease is a series of rust-like colored bumps on the underside of the leaves. There are previous infection reports in India, Nigeria, Louisiana, and Malaysia (Baiswar *et al.*, 2015; Hernandez *et al.*, 2005; Holcomb & Aime, 2010). Despite these reports regarding the presence of *Plumeria* rust on *Plumeria rubra* around the world, we found only one recorded instance of this disease in Puerto Rico (Roure, 1962). It is possible that this lack of attention is due to a lack of information on the biomedical potential of the host, which has only been reported recently (Mistry *et al.*, 2014). Therefore, to the best of this investigator's knowledge, the present work is the second report of *C. plumeriae* infecting *P. rubra* in Puerto Rico.

The genus *Coleosporium* is part of the *Coleosporaceae* family and *Pucciniales* order. The family alone contains over 80 similar species (Weeraratne & Adikaram, 2006). The infection of the host by *C. plumeriae* results in an early abscission of the leaves from the tree branches, eventually resulting in death (Wang *et al.*, 2011). An example of the symptomology of the rust fungus on *P. rubra* is shown in **Figure 5**.

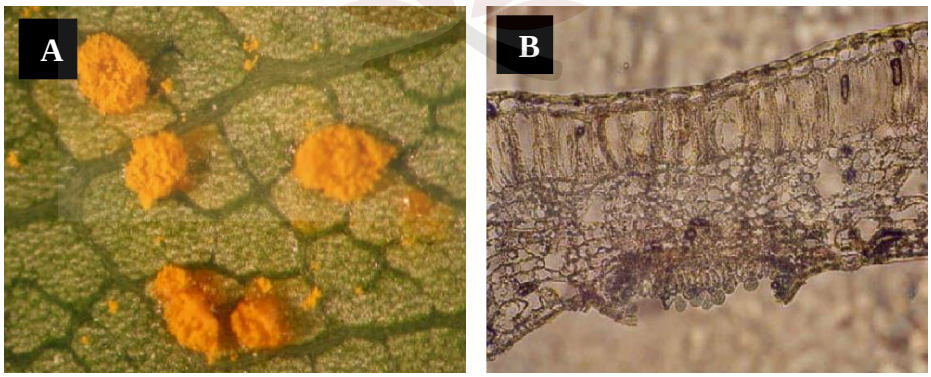


Figure 5*: Characteristics of Coleosporium plumeriae on Plumeria rubra* (**A**) Uredia of *Coleosporium plumeriae* on *Plumeria rubra* (30x), (**B**) Transverse section of a uredium at 100x. Adapted from (Weeraratne & Adikaram, 2006).

The symptoms of *C. plumeriae* have been described rather uniformly worldwide (Baiswar *et al.*, 2015; Hernandez *et al.*, 2005; Holcomb & Aime, 2010). It is usually characterized by small, rounded, raised and orange pustules called uredinia found on the underside of leaves. On the top

side of the leaf, the uredinia cause yellow spots that are spaced throughout the leaves. The longer the disease prevails on the leaf, the yellow spots can take on a brownish, necrotic aspect (Nelson, 2009). The pustules can range between 0.5mm to 1.5 mm in size (Weeraratne & Adikaram, 2006). Once the leaves get to that browning stage, they can begin to dry and curl up until they are abscessed prematurely; early abscission rates can reach up to 100%, as a result of all the leaves falling off the affected tree (Nelson, 2009). However, the rust does not infect the stems or flowers, just the foliage. The infection is spread in the wind when urediniospores from infected plants travel until they stick to the underside of a healthy leaf under wet or humid conditions (Nelson, 2009). The spores then grow, penetrate the leaf surface where they continue to grow more hyphae which in turn infect the cells of the leaf. Once they are mature, these spores erupt back out on to the leaf surface and create the orange, powdery pustules as well as the lesions on the back of the affected leaf seen in **Figure 6** (Nelson, 2009). These visible signs of infection facilitated the identification of infected trees and leaves for the purpose of determining prevalence and incidence, respectively. Furthermore, easy identification also facilitated sample collection for genetic analyses.

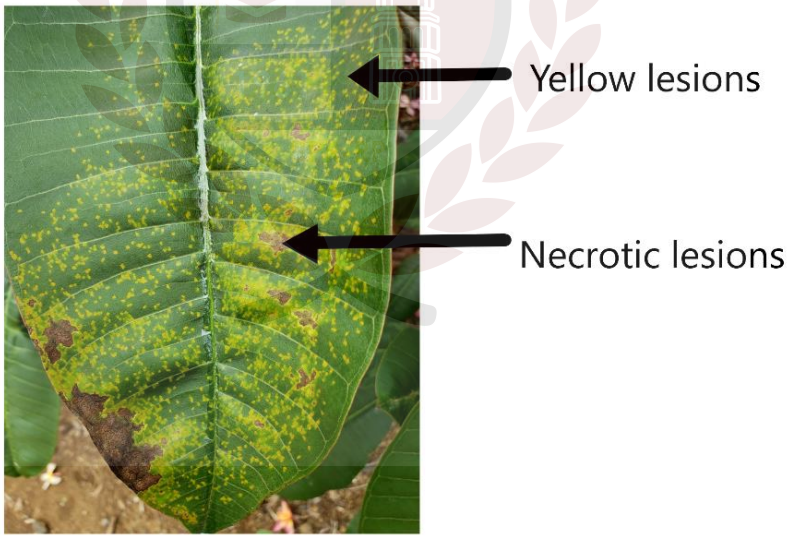


Figure 6: *Coleosporium plumeriae on Plumeria leaf.* Yellow spotting and necrotic damage on the top of the leaf is an indicator of disease on *Plumeria* spp. Adapted from (Nelson, 2009).

C. plumeriae has a grave impact on its host and the environment. However, this impact is not instantaneous, therefore, it is possible to take action against the disease. To act against an invasive pathogen, the more we know about it, the better we can control it. **Figure 7** shows the well-known pattern of invasiveness (Sakai *et al.*, 2001). This entire series of events must occur for

an invasive species to have a negative effect on its surroundings. Worldwide, *C. plumeriae* is currently in its Control/Restoration stage because it has begun to have a negative impact in ecological terms (Hernandez *et al.*, 2005; Nelson, 2009; Oliveira *et al.*, 2019; Yang *et al.*, 2014).

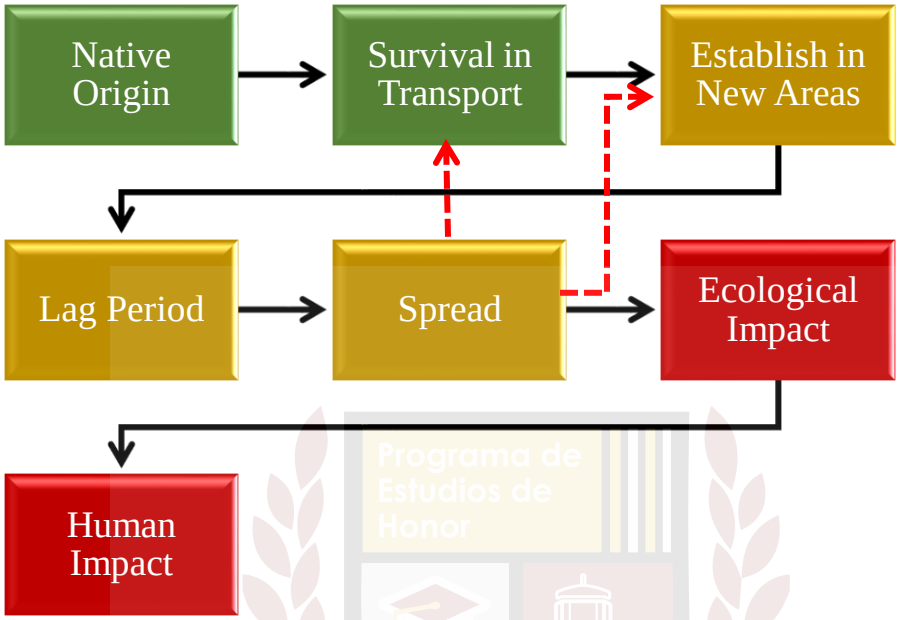


Figure 7: *Invasive species impact process in relation to their management*. An invasive species begins in a native site, and is later transported to a new area. The first two steps (labeled green) represent the phase at which an invasive species is in the Prevention stage. The following three steps (labeled yellow) represent the successful establishment, lag and spread of an invasive species, therefore moving into an Eradication stage. In the spread stage, the species can once again transport itself to another area and establish there as well (represented with red arrows). The final two steps in an invasion correspond to the Ecological and Human impact (labeled red), which represent the Control and/or Restoration stage. Adapted from (Sakai *et al.*, 2001).

To control the spread of an invasive pathogen at the Control and Restoration stage, the pathogen's reproductive and infectious stages must be elucidated (Sakai *et al.*, 2001). This requires the answers to questions such as: How does this pathogen impact biodiversity?, Does it have the potential for rapid evolution?, and What traits of the pathogen allow for predictions to be made in order to prevent its spread? (Sakai *et al.*, 2001). These questions can often be answered by a variety of studies. Those studies should aim to elucidate the pathogen's genetic diversity, reproduction and rate of spread. This information can be obtained from a variety of avenues, including, but not limited to, genetic sequencing, incidence and severity studies, epigenetics, and ecological surveys (Perez *et al.*, 2015). Our study contributes to answering these big questions for *C. plumeriae* by providing data on prevalence to address biodiversity, data on phylogeny for potential longitudinal studies about the fungus's spread and data on incidence to inform spread preventative measures.

An overall benefit of studying invasive species, beyond protecting their hosts, is their great potential as a model for evolutionary biology. Once a species invades a new area, they must undergo changes to better adapt to their environment. This makes invasive species an ideal model to study rapid evolutionary change, as well as their interactions with natural selection and native species (Sakai *et al.*, 2001). Additionally, their exposure to new selective pressures and interactions with native species provide evolutionary biologists with a unique opportunity to study the rate and predictability of evolution in the wild (Huey *et al.*, 2005). Furthermore, the restoration aspect also takes into consideration the potential negative human impact that the spread of the pathogen on a certain host might have and studies to mitigate against any lasting damage (Sakai et al., 2001). For example, a fungus that infects important food crops, such as wheat, can pose a threat to human food security if accidentally introduced to any of the major wheat producing areas. Studying the genetic diversity of an invasive fungus helps us understand more than just its evolutionary change. Genetic diversity studies provide better insight into modes of reproduction, particularly whether a fungus reproduces sexually (Hassine *et al.*, 2019). In this study of *C. plumeriae*, our interest lies in elucidating its prevalence, incidence and genetic diversity to better control its spread in Puerto Rico and to protect *Plumeria* for their therapeutic benefit.

***Plumeria Rust*: Reproduction, Spread and Control**

According to previous works, *Coleosporium* species are generally heteroecious (Weeraratne & Adikaram, 2006). This means that the fungus's spermogonia and aecia spore states occur on one host, while its remaining spore states live on another host (Yamaoka, 2014). A study conducted in 2006 stated that they had identified *Coleosporium* spp.'s aecia and spermogonia on *Pinus* needles, and *Coleosporium* spp.'s basidia, uredia and telia on both monocots and dicots (Weeraratne & Adikaram, 2006). However, they make no mention of *C. plumeriae* specifically because its mode of reproduction is still unknown. But we do know that at least some members of the genus can reproduce on *Pinus* needles. This fact adds credence to our hypothesis for a possible introduction of *C. plumeriae* to Puerto Rico on imported *Pinus* trees.

According to a Hawaii Department of Agriculture report, *C. plumeriae* is adaptable to its environment over time and it is hypothesized this is due to an ability to adapt to environmental conditions using sexual reproduction to produce more varied progeny (Nelson, 2009). It is unclear if this adaptability reported in 2009 pertains to the specific Hawaii strains or if to all *C. plumeriae*

strains. The author goes on to state that the spermagonial and aecial stages of the fungus are unknown, which has been corroborated by many other studies (Holcomb & Aime, 2010; Kumar & Khurana, 2016; Oliveira *et al.*, 2019; Yamaoka, 2014). Additionally, *Plumeria* trees that are well-kept and have their infected or fallen leaves removed have been shown to be less likely to promote the spread of the leaf rust (Nelson, 2009).

As with other rust fungi, *C. plumeriae* has extremely limited growth in culture. This has made studying the fungus significantly harder. However, one way to get around this obstacle to observe the spread of the disease by inoculating living host plants. A study in Louisiana conducted pathogenicity tests by spraying healthy *Plumeria* leaves with the fungus and the rest with water as a control (Holcomb & Aime, 2010). They found that within 10 days, the leaves sprayed with the *C. plumeriae* inoculum were infected (Holcomb & Aime, 2010). While the fungus was not actually cultivated in a traditional sense in this experiment, the *C. plumeriae* inoculation method carried out by these investigators shed some light on a possible method for *in vivo* culture of the fungus, and gathered information regarding the speed of infection and its severity (Holcomb & Aime, 2010).

Additional to its difficult cultivation, there is also little known regarding the sexual reproduction of *C. plumeriae*. Due to the inability to cultivate the spores *in vitro*, there have been no experimental studies that show the fungus reproduces sexually, and the sexual stage has never been observed in nature. This study aims to determine the genetic diversity of *C. plumeriae* which may provide information regarding its reproduction. For example, as an invasive species, if *C. plumeriae* was introduced to Puerto Rico only once and does not reproduce sexually, the fungus's genetic diversity should be minimal. However, if sexual reproduction occurs (or if there were multiple introductions) the genetic diversity should be greater. Determining precisely which of the two alternatives is true is beyond the scope of this Jr. Thesis, a study limited to prevalence, incidence and genetic diversity.

Geographic Range of *C. plumeriae*

Several researchers have studied the spread of this fungus across different corners of the world. Since *C. plumeriae* was first reported in 1852 (over 150 years ago), in the Dominican Republic, it was said to have a potential to spread over tropical and sub-tropical America, which it did until the 1980s when it spread beyond those regions to places as far as China and Vietnam

(Wang *et al.*, 2011). *C. plumeriae* is also present in Africa, Taiwan and Hawaii (Chung *et al.*, 2006; Hernandez *et al.*, 2005; Nelson, 2009; Wang *et al.*, 2011). This range jump suggests the possible influence of human travel on the dissemination of the fungus, a phenomenon we have been aware of since as early as the 1700s (Wilson, 2012). For example, when one considers that the fungus's genus is heteroecious with some of its spore states existing on *Pinus* needles, it may be that the *Coleosporium* rust disease was spread from the Dominican Republic on imported *Pinus* trees (Durland, 1922). This hypothesis sustains ideas on the origin of this fungus in Puerto Rico because native *Pinus* species from Hispaniola have been introduced to the island (Acevedo-Rodríguez & Strong, 2005). Further study into the genetic diversity of this fungus could potentially shed some light on this theory. **Figure 8** shows the reports of *C. plumeriae* on *Plumeria alba* or *Plumeria rubra* worldwide.

***Figure 8**: Coleosporium plumeriae Infection Spread on Plumeria spp*. The red circle represents original distribution of the disease emergence. Adapted from reports from (Baiswar *et al.*, 2015; Chung *et al.*, 2006; Hernandez *et al.*, 2005; Holcomb & Aime, 2010; Kakishima *et al.*, 2017; Nelson, 2009; Salazar Yepes & Alves de Carvalho Junior, 2013; To-Anun *et al.*, 2004; Wang *et al.*, 2011).

Studies of genetic diversity often provide information that is critical in the control of fungal pathogens. Genetic diversity has been previously linked to pathogenicity. A recent study (Chappell & Rausher, 2016) provided a challenge to the classic one pathogen-one host theory. The authors discovered that while pathogen genotypes within a local community tend to be host-specific, the same pathogen genotypes can be pathogenic to more than two or three hosts from outside

communities. They tested this by inoculating introduced (invasive) plants with a local rust fungus in the lab and observing whether infection occurred (Chappell & Rausher, 2016). This further supports the hypothesis that once introduced, *C. plumeriae* could possibly spread to new host species.

In order to determine the different factors contributing to the species diversity of *C. plumeriae* in Puerto Rico, the prevalence and incidence of the pathogen was evaluated. Prevalence of a fungal pathogen is considered the proportion of trees that are infected with the fungus versus the total number of trees in the convenience sample, as expressed in the Project Overview. The incidence is evaluated as the proportion of infected leaves versus healthy leaves per branch.

In a 2014 study in La Habana, Cuba, researchers found *C. plumeriae* on both *P. rubra* and *P. obtusa*. 77.6% of *P. rubra* plants surveyed were infected (Martinez de la Parte & Garcia-Rodriguez, 2014). *P. obtusa* is rarely found in La Habana, yet 2 out of the 4 plants surveyed were infected as well. The authors note that the infection's severity varied and that in the most advanced cases, they observed severe necrotic damage to the affected leaves (Martinez de la Parte & Garcia-Rodriguez, 2014). A similar study conducted in Guangdong Province, China found that the prevalence of *C. plumeriae* was 91% in *P. rubra* plants, with the most severe infections resulting in necrosis (Yang *et al.*, 2014).

Environmental Effects on *Plumeria* Rust Spread

C. plumeriae was first recorded in 1852 in the Dominican Republic under the name *Uredo domingensis* (Kakishima *et al.*, 2017). From there, it spread mostly to tropical and subtropical areas of Central America. In the case of Puerto Rico, the *Plumeria* rust was first reported as *Coleosporium domingensis* on *P. rubra* in 1920, and on *P. alba* on 1959 (Roure, 1962; To-Anun *et al.*, 2004). *Coleosporium domingensis* was later found to be a synonym for *C. plumeriae* by Arthur Berk in 1918 (Roure, 1962; To-Anun *et al.*, 2004). To our knowledge, there have been no recent publications regarding *Plumeria* rust infecting either of these hosts on the island.

Plumeria grow well in hot, dry areas which makes tropical and sub-tropical islands ideal for these hosts. In Hawaii, *Plumeria* is grown at an average elevation of 600 meters. The most common species in Hawaii are *Plumeria obtusa* and *P. rubra*, both affected by *Plumeria* leaf rust since 1991 (Nelson, 2009). Yet some of the rarer species on the island seem to be more resistant to the effects of the *Plumeria* leaf rust. Among the moderately resistant species that are kept in the

College of Tropical Agriculture & Human Resources (UH-CTAHR) is *P. alba*, one of the other species present in Puerto Rico (Nelson, 2009).

It is currently unclear which environmental conditions favor *Coleosporium plumeriae* infection of *Plumeria*. However, a previous study has hypothesized that the worldwide distribution of this pathogen may be related to the ecological events El Niño and La Niña. The El Niño-Southern Oscillation (ENSO) cycle is a meteorological phenomenon that consists of two opposite events known as El Niño and La Niña (National & Atmospheric Administration, 2020). The term is used to describe temperature and atmospheric fluctuations in the Pacific Ocean. El Niño is known as the warm phase of ENSO and La Niña is known as the cool phase (National & Atmospheric Administration, 2020). These events can occur every 2-7 years, can usually last up to 9-12 months and result in intense periods of dryness, high temperatures and strong winds in the case of El Niño, in contrast to wetness, low temperatures and strong/moderate winds during La Niña (National & Atmospheric Administration, 2020). Due to *Coleosporium plumeriae' s* preference for humid environments, it stands to reason that La Niña events are more favorable to the fungus's growth.

From 1988-1989 there was a registered peak in La Niña activity which lead to strong winds and moist/humid conditions (Kakishima *et al.*, 2017). This led investigators to hypothesize that the pathogen was spread across the Pacific Ocean due to these strong ecological conditions (Kakishima *et al.*, 2017). have been reported instances of the spread of other pathogen due to meteorological events. For example, as recently as 2016, El Niño events were related to the resurgence of certain human diseases such as cholera and malaria, while La Niña has been related to a stronger reemergence of influenza, dengue and yellow fever (Flahault *et al.*, 2016).

In the present study, we conducted a brief ecological survey where both the growing conditions and the locations of each specimen was recorded. Among the environmental conditions that were surveyed are host species, elevation, exposure to sunlight and presence of herbivores.

Raman spectroscopy Analysis of Rust Spores

Spores can be identified in several ways. Among these are microscopic analysis, genetic analysis and Raman spectroscopy. The microscopic and genetic analysis procedures are explained in detail in the methodology section. While microscopic and genetic analysis are fairly common

in this field, Raman spectroscopy is a relatively recent addition. For this reason, we deem it necessary to explain its relevance to our study.

Raman spectroscopy was invented in 1928 by Sir Chandrasekhara Venkata Raman when the only instrumentation available to him was sunlight and a telescope (Ferraro *et al.*, 2003). Today, Raman has evolved to be a highly trustworthy technique with a wide set of applications within and outside Chemistry. A laser of a pre-determined wavelength within the UV visible range is aimed at a sample and said sample is excited into a vibration transition state that is then reflected and absorbed by the instrument at an angle perpendicular to the laser incidence (Ferraro *et al.*, 2003). With these measurements, the instrument displays a Raman spectrum. By aiming this laser at the sample for a certain period of time – for example: 30-60 seconds– the instrument can record any changes in that vibration transition state and translate it into a Raman shift, which we observe as peaks in the Raman spectra (Ferraro *et al.*, 2003). These peaks correspond to specific molecular bond vibrations which allow us to make chemical structure comparisons across samples (Ferraro *et al.*, 2003).

a 2012 study demonstrated that it was possible to identify fungal spores obtained from air vents and water pipes using Raman spectroscopy(Ghosal et al., 2012. They compared the signatures obtained from single spores to those of a database they prepared with common fungal spores found in those environments. Their hope was to be able to identify unknown samples to a relatively high degree of certainty using Raman spectra. Their results suggest that reliable Raman spectra can be obtained from individual spores and that each species provides a distinct enough spectra to be easily distinguished from other fungi (Ghosal *et al.*, 2012).

The Raman spectroscopy technique was useful to us in a somewhat similar application. Raman spectra were obtained for some of our leaf rust samples. The spectra demonstrated significant variability between spore samples from different locations or different hosts. Our aim at the time was to use this technique to add reliability to our phylogenetic trees for genetic analysis.

The most definitive way fungal spores can be identified is through genetic and phylogenetic analysis. Phylogeny is the study of evolutionary relationships between different organisms (Willey *et al.*, 2013). Phylogenetic trees serve as a visual model to represent hypothetical evolutionary relationships between groups of interest. These trees can be constructed from associations between the genetic sequences of certain organisms at a specific site (Sarma *et al.*, 2018). Learning more

about *C. plumeriae's* diversity contributes to the study of basic sciences and population biology (Sakai *et al.*, 2001).

There are theories regarding the evolution of *C. plumeriae*. A study in Malaysia determined that *C. plumeriae* was most likely phylogenetically related to *Coleosporium solidaginis*, a rust that affects *Solidago* species in North America (Oliveira *et al.*, 2019). Oliveira et al. (2019) used the internal transcribed spacer (ITS) region to compare species in GenBank. An example of the leaf rust's phylogenetic tree is shown in **Figure 9**.

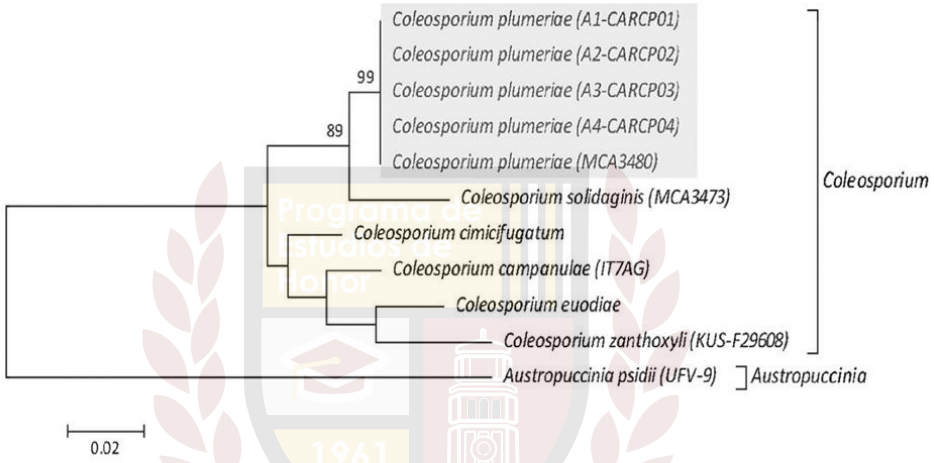


***Figure 9**: Coleosporium plumeriae of Malaysia Phylogenetic Analysis*. Sequenced ITS region with primers ITS 1 and ITS 4 and compared sequences with those in the GenBank. Adapted from (Oliveira *et al.*, 2019).

The Oliveira study included an inoculation study with healthy host plants to determine whether *C. plumeriae* followed Koch's postulates for obligate parasites. The postulates are four: 1) the microorganism must be present in all diseased organisms, but absent in healthy control organisms, 2) the suspected microorganisms must be isolated and grown in culture, 3) when a healthy host is inoculated with the isolated microorganism, the same disease must arise and, finally, 4) the same microorganisms must be isolated from the newly inoculated host (Willey *et al.*, 2013). The plants that were inoculated in the lab by Oliveira's group showed the same symptoms after 4.5 days as the original samples collected in the field. Once they compared their DNA sequences and found them to be identical, the researchers verified that their fungus met Koch's postulates successfully. Therefore, they confidently concluded that their method of spraying the spores on healthy plants lead to the same infection (Oliveira *et al.*, 2019). The relationship between *C. plumeriae* in Puerto Rico and isolates in other countries is currently unclear. This work aims to dilucidate this relationship through phylogenetic analysis.

Alternatives to Control *Plumeria* Rust Spread

It is important to control the spread of this pathogen without impacting the medicinal benefits of the host nor endangering the environment because of the toxicity of the fungicides used to control its spread. Systemic fungicides are very toxic and function by being fully absorbed into the plants they are spread on (McGrath, 2004). Non-systemic fungicides are not fully absorbed into the plant tissues and are often used at early stages of infection (Dreikorn & Owen, 2000). Both classes of fungicides do harm to humans, animals, and the environment. There are two main fungicide strategies that can be employed against rusts: protectants (a variation of non-systemic fungicides) and systemic fungicides. In this section, we explore the noxious and potentially lethal effects of these fungicides. Our study contributes phylogenetic and incidence information which provides a better understanding of this rust fungus so that, in the future, more ecologically safe measures can be taken to protect its host and control its spread.

Protectant products are a method of chemically controlling a fungal pathogen before the infection has taken hold of a host (as a preventative measure). This is because protectants do not penetrate the plants they treat. Some protectant chemicals have been effectively used against Plumeria rust, such as chlorothalonil, mancozeb and sulfur (Australia, 2016). However, none of these protectants come without a cost. In the case of chlorothalonil, several works have shown its toxicity to animals, plants and sea life. It was also been classified as a probable human carcinogen and has been shown to have a negative impact on soil systems and diversity (Caux *et al.*, 1996; Chen *et al.*, 2001).

Mancozeb was a popular, broad-spectrum, dithiocarbonate-based fungicide that was commonly used to treat fungal pathogens in the early 2000s (Gullino *et al.*, 2010). It has been used as a protectant against Plumeria, Corn and Stone fruit rusts, along with a large number of fungal plant pathogens (Australia, 2016; Gullino *et al.*, 2010). Yet, mancozeb is highly toxic to humans and animals and has been shown to cause adverse and potentially lethal health effects whether ingested, smeared directly on skin or sprayed as an aerosol (Gullino *et al.*, 2010).

Another, non-systemic, protectant fungicide that is commonly used against Plumeria rust is sulfur. Elemental sulfur is one of the oldest fungicides in the world, possibly going back as far as the ancient Greeks (Tweedy, 1981). This fungicide has been used against a broad range of fungi, as well as an active ingredient in several fungicide mixtures. However, elemental sulfur has been

linked to several toxicity issues and can penetrate and remain in diverse soils, negatively impacting soil diversity (Kuklińska *et al.*, 2013).

Officials in Hawaii have determined a range of systemic fungicides that destroy *Plumeria* rust (Nelson, 2009). These products include: Eagle 40WP, Eagle 20EW and Heritage. The active ingredients in these fungicides are myclobutanil and azoxystrobin in different formulations (Nelson, 2009). These are currently used in greenhouses and plant nurseries to keep the pathogen at bay.

Myclobutanil is a common fungicide used against different kinds of fungi. Its mode of action is to inhibit the production of Ergosterol (Loso *et al.*, 2018). Myclobutanil is commonly used in both the United States and the European Union as a fungicide for Ascomycetes and Basidiomycetes. It is moderately toxic if ingested and can be moderately toxic to the soil and fish (Pesticide Properties, 2019b). The chemical structure for Myclobutanil is shown in **Figure 10-A**.

Azoxystrobin is a broad spectrum strobilurin fungicide approved for use in both the US and EU (Nelson, 2009). Its most common use is for controlling fungal pests in cereals and wheat rusts. It is effective against other rusts and is used in Hawaii to control *C. plumeriae* (Nelson, 2009). The mode of action for Azoxystrobin is to inhibit respiration processes in the rust mitochondria, making it unable to produce energy. This is more commonly known as an QoL type fungicide (Pesticide Properties, 2019a). This fungicide has a low toxicity, but does has the potential to bioaccumulate and cause problems when ingested regularly (Pesticide Properties, 2019a). The chemical structure for Azoxystrobin can be found in **Figure 10-B**.

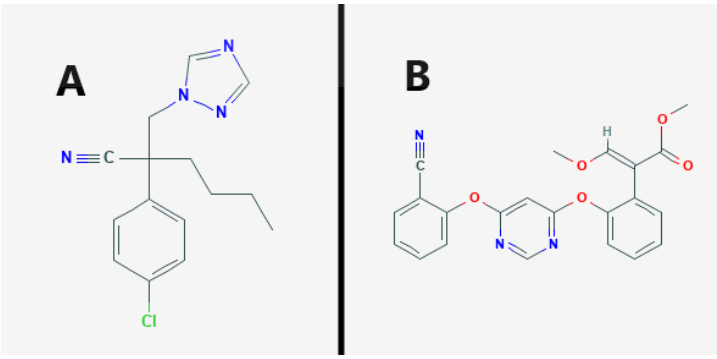


***Figure 10**: Fungicide Active Ingredient Chemical Structures.* A) Structure of Myclobutanil PubChem CID: 6336, B) Structure of Azoxystrobin PubChem CID: 3034285. Structure information obtained from PubChem.

Other systemic fungicides used against *Plumeria* rust include: oxycarboxin and triadimefon (Australia, 2016; McMillan, 1984). The use of Oxycarboxin as a fungicide was first reported in 1984 and demonstrated promising capabilities to control the spread of the fungal infection. It was also reported to be effective when controlling defoliation in both plant nurseries and fields (McMillan, 1984). The mode of action of this fungicide is the inhibition of nucleic acid synthesis. However, this pesticide was banned in the United States due to its high toxicity of animals and soil (Pesticide Properties, 2020).

Similarly, the systemic fungicide triadimefon is commonly used against a variety of fungal pathogens of ornamentals, fruits and agricultural crops (Australia, 2016; Jiang *et al.*, 2017). The widespread use of triadimefon in agricultural industry has been linked as a significant risk factor in the pesticide's run-off impacts on marine ecosystems. This fungicide has been linked to possible carcinogenic effects, neurotoxicity and reproductive toxicity in humans (Jiang *et al.*, 2017; Martin *et al.*, 2007; Menegola *et al.*, 2000).

Therefore, both protectants (chlorothalonil, mancozeb and sulfur) and systemic chemicals (oxycarboxin and triadimefon) are fungicide alternatives that are capable of controlling *Plumeria rust*, but present significant risks and limitations in terms of toxicity to humans, animals and marine ecosystems. While these methods can control the spread of the pathogen, they are unable to do so without resulting in noxious and toxic effects on humans, animals and the environment. The question of how to control *C. plumeriae* without contributing to toxicity remains unanswered. It is our hope that the more we know about the fungus the better able to develop an environmentally friendly way to protect the *Plumeria* host and its medicinal and aesthetic benefits.

***Plumeria spp.*: Therapeutic Benefits**

The genus *Plumeria* is known for its shrubs and ornamental trees which are commonly cultivated for ornamental use in tropical regions (Choudhary *et al.*, 2014). Said trees are also commonly used in religious ceremonies in India and Hawaii, and have recently gained recognition for their potential use in biomedical applications, mostly due to the latex of their leaves. These include uses such as fighting against infections, anti-inflammatory agents, antioxidant properties and as an anti-tumor agent (Choudhary *et al.*, 2014).

Currently, many *Plumeria* species are being used as purgatives or to help treat asthma, rheumatoid arthritis, gonorrhea and blood disorders (Choudhary *et al.*, 2014). *P. rubra* flowers are

reportedly commonly used in Malaysia by local medical practitioners as a remedy for arthritis-related diseases (Mohamed Isa *et al.*, 2018).

Our primary host of interest in this work is *Plumeria rubra* known commonly as the Red Aleli o Red Frangipani. Of the Apocynaceae family, this tree is native to southern Mexico and Central America (south to Costa Rica), but largely naturalized in Puerto Rico (Little & Wadsworth, 1964).

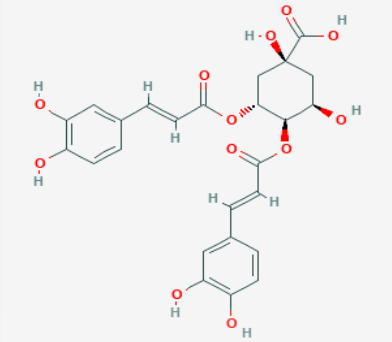
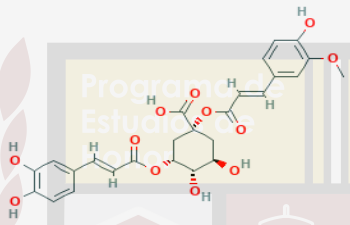
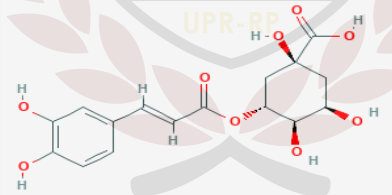
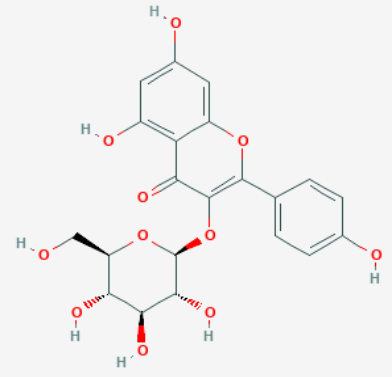
We are also interested in the White Aleli or White Frangipani, *Plumeria alba. P. alba* is a small tree that grows commonly in dry forests and coastlines around Puerto Rico. *P. alba* is known for the latex-like substance produced by its leaves. The leaves can grow up to 30cm long and this tree is known for its beautiful white flowers (Sloan *et al.*, 2007). At first glance, both trees share several similarities in their overall morphology. However, the difference is that *P. rubra* was introduced from Mexico, whereas *P. alba* is native to Puerto Rico (Little & Wadsworth, 1964). It was unknown whether *C. plumeriae* affected *P. alba* in Puerto Rico, because this host species was reported as being moderately resistant to infection in Hawaii (Nelson, 2009). This was evaluated in this inquiry on prevalence and incidence and we found that *P. alba* was indeed affected by *C. plumeriae* in Puerto Rico, though rarely.

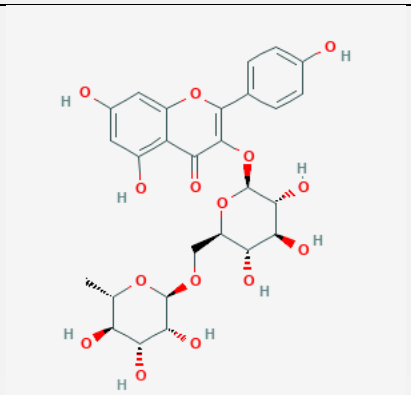
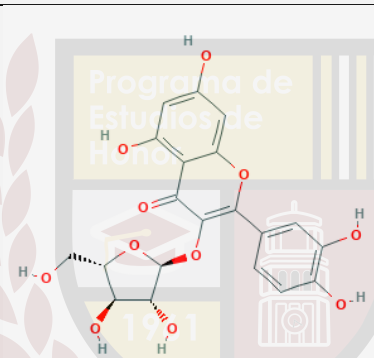
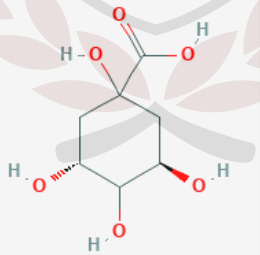
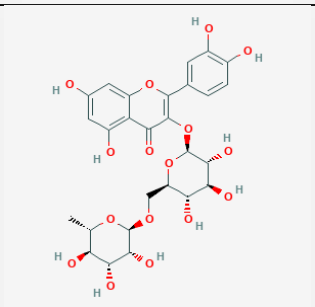
A Malaysian study conducted *in vitro* and *in vivo* analysis to identify which compounds of the *P. rubra* flower held the therapeutic benefits and their mode of action. Compounds were identified with Liquid Chromatography and tandem Mass Spectroscopy (LCMS/MS). They detected the following compounds: 3-O-caffeoyolquinic acid, 5-caffeoquinic acid, chlorogenic acid, citric acid, kaempferol-3-O-glucoside, kaempferol-3-rutinoside, kaempferol, quercetin 3-O-α-L-arabinopyranoside, quercetin, quinic acid and rutin (Mohamed Isa *et al.*, 2018). Each of these compounds has been linked to a potential biomedical application, as shown in **Table 1**. Additionally, the researchers found the flower contained high levels of active phytochemical compounds that strongly inhibit redox activity *in vitro* and *in vivo* by attacking free radicals to reduce oxidative stress (Mohamed Isa *et al.*, 2018).

P. alba also has some medicinal applications (Baiswar *et al.*, 2015). A recent chemical study evaluated the antioxidant and phenolic properties of methanolic flower extracts of these trees. They found that *Plumeria* species may become new source of useful drugs in the treatment of colon cancer (Mistry *et al.*, 2014).

Specifically, Mistry et al. found that 98% methanol extracts of flowers had relatively pure antioxidant and phenolic compounds. They subjected the methanol extracts to several assays such as the 2, 2'-diphenyl-2-picrylhydrazyl (DPPH) assay to test for anti-oxidative stress, the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay to test for cancer cell cytotoxicity, and anti-cholesterol assays. The most promising aspect of the study was *P. alba's* significant cytotoxicity and antiproliferative action against colon cancer cell line HCT 116. *P. rubra* was found to have significant hypolipidemic activity *in vitro*, making it an excellent candidate for treating hyperlipidemia, a major risk factor of coronary artery diseases. This is just one example of the pharmaceutical and biomedical importance of both *Plumeria* species and further reason to conserve and encourage their growth on our Island.

***Table 1**: Biomedical Applications for Plumeria rubra Compounds*

Compound	Structure	Medical Properties	References
3-O-caffeoyolquinic acid		Antiviral against respiratory syncytial virus (RSV)	(Li *et al.*, 2005) PubChem CID: 5281780
5-caffeoquinic acid		Suppress P-selectin expression on mouse platelets when orally administered.	(Park, 2009) PubChem CID: 131753173
Chlorogenic acid		Potential to protect against neurological degeneration associated with oxidative stress in the brain	(Veljkovic *et al.*, 2018) PubChem CID: 1794427
Kaempferol-3-O-glucoside		Potentiates migration of HaCaT cells, good potential for wound healing	(Petpiroon *et al.*, 2015) PubChem CID: 5282102

Compound	Structure	Medical Properties	References
Kaempferol-3-rutinoside		Promotes keratinocyte migration through FAK and Rac1 activation	(Petpiroon *et al.*, 2015) PubChem CID: 5318767
Quercetin 3-O-α-L-arabinopyranoside		Potentiates preventive effects of stress-induced gastritis	(Park *et al.*, 2018) PubChem CID: 5490064
Quinic acid		Antioxidant metabolism increases urinary excretion of Tryptophan and Nicotinamide	(Pero *et al.*, 2009) PubChem CID: 6508
Rutin		Antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities	(Ganeshpurkar & Saluja, 2017) PubChem CID: 5280805

All of these biomedically-relevant compounds are potentially at risk because of the rust fungus's threat to *Plumeria* species' health. (Voegele *et al.*, 2009) showed that rust fungi infections cause significant metabolic and photosynthetic changes in their hosts (Voegele, Hahn, & Mendgen, 2009). These include changes in carbohydrate distribution, metabolic adaptations for defense against infections and an overall decrease in chlorophyll content in affected leaves (Voegele *et al.*, 2009). All of these changes create challenges for the plant to be able to produce energy, which in turn may cause a deficiency in non-essential compound production in the leaf. Their study implies that the aforementioned medicinal phenolic compounds may be threatened by rust diseases.

The other *Plumeria* species present in Puerto Rico include *Plumeria obtusa* and *Plumeria púdica*. *P. obtusa* is similar in height to *P. rubra*, but it is known for having much more round-shaped leaves when compared to *P. rubra's* slightly pointed ones (Corner, 1997; Kamariah *et al.*, 1999). Previous work has found that *P. obtusa* might have potential for use in the essential oils industry (Kamariah *et al.*, 1999). *P. púdica* is native to Colombia, Panama and Venezuela, but often used as an ornamental tree in the Caribbean thanks to its long blooming period. *P. púdica* is distinguished for its pointed, arrow-shaped leaves and its white flowers (Suarez *et al.*, 2017). Though revered for its ornamental beauty, like the rest of the *Plumeria* species discussed, *P. púdica* has been recorded as having beneficial therapeutic effects as well. These include applications as a carminative, laxative, diuretic, as well as an anti-inflammatory, anti-microbial and anti-ulcer agent (Chatterjee *et al.*, 1991; Vijayalakshmi *et al.*, 2011). *P. púdica* has even been cited in some old literary texts as being useful as a treatment against certain skin diseases such as leprosy (Chatterjee *et al.*, 1991). In short, the medicinal content of *Plumeria* species in Puerto Rico necessitates a broader interest in its preservation and protection from diseases such as *C. plumeriae*. My work can be used to inform an ecologically sound solution to protect *Plumeria* from *C. plumeriae*.

A *Plumeria* Predator: *Pseudosphinx tetrio,* the *Plumeria* caterpillar

P. rubra and *P. alba* are also known as the "wormy tree" due to being a preferred host for large larvae of *Pseudosphinx tetrio*, better known as the *Plumeria* caterpillar. These caterpillars are *Plumeria* pests and must be considered in the study of *Plumeria spp.* since they consume a significant amount of the tree's leaves, inflorescences, fruits and flowers (Sloan *et al.*, 2007). This is relevant to our study because *P. tetrio's* eating of infected leaves can have a direct impact on disease incidence (measured by counting the number of infected vs total leaves in a branch). It is

also possible that *P. tetrio* may serve as a potential disease vector for *C. plumeriae*, spreading the spores from infected leaves to healthy ones by walking on the tree. This would also have a potential impact on disease incidence and prevalence, since these caterpillars not only walk across tree branches, but also from one tree to the next (Dunford & Barbara, 2011; Sloan *et al.*, 2007).

The *P. tetrio* caterpillar is found in tropical and subtropical America, and has been commonly associated with both *P. alba* and *P. rubra* (Dunford & Barbara, 2011). The larvae have been known to seriously affect *P. rubra* trees from July to September (Santiago-Blay, 1985). It is also speculated that the caterpillar is capable of using the latex produced by *P. alba's* leaves in order to protect itself from predators (Dunford & Barbara, 2011). These caterpillars commonly appear when the trees are kept in gardens or areas with consistent landscaping and they can completely defoliate an entire tree in a matter of days. A single caterpillar can eat up to three big leaves in a single day (Dunford & Barbara, 2011). Such a close interaction between this caterpillar and the tree makes the insect a potential dispersal agent for the *C. plumeriae* to spread from one tree to another. An example of *Pseudosphinx tetrio* on a nearly defoliated *Plumeria* can be found in **Figure 11**.

Figure 11*: Pseudosphinx tetrio on Plumeria spp.* Specimen in Old San Juan, Puerto Rico had more than 20 caterpillars. Some in this picture are consuming the tree's stem, a behavior carried out once there is a lack of leaves. Picture taken by author, Melanie Ortiz.

Insects that have close associations with the plants they live in can occasionally serve as a dispersal agent for that plant's diseases. The close proximity of a fungus to an insect may provide it with the opportunity to transport into a place it wasn't able to reach originally, or provide a new access point for it to overcome the host's defenses (Vega *et al.*, 1999). A possible reason for this interaction could be that the fungus serves as food for the insect, or perhaps as a mechanism to make the host plant more easily digestible for the insect. Most of these fungal and insect relationships tend to be mutualistic in nature; they both tend to benefit from the arrangement. However, some parasitic and commensal (one-sided benefit with neutral effect on the other) relationships have also been reported in literature (Vega *et al.*, 1999). One example of insects acting as dispersal agents in rusts was observed in 2002, when it was determined that the aphid *Uroleucon cirsii* and the beetle *Cassida rubiginosa* both helped increased fungal dispersion rates in the weed rust *Puccinia punctiformis*. They were able to do this through mechanically distributing spores and overall increasing the number of infected tissues (Kluth *et al.*, 2002). We speculate that *P. tetrio* may have a similar mutualistic interaction with *Plumeria* rust. For these reasons, as well as evaluating the genetic diversity, prevalence and incidence of *C. plumeriae* due to ecological factors on *P. alba/rubra*, this study will also take into consideration the presence/absence of the *Pseudosphinx tetrio* caterpillar on each of the specimens evaluated.

Section 2: Methodology

Aim 1: Evaluate the prevalence and incidence of *C. plumeriae* on *Plumeria* spp. in Puerto Rico and determine the impact of ecological factors on both prevalence and incidence.

Hypothesis: We hypothesized that *C. plumeriae* distribution would vary due to environmental conditions among the *Plumeria* spp. on the Island and that those growing in shade would have a higher infection incidence. *C. plumeriae* would be more prevalent among the *Plumeria* spp. in humid environments, since the windborne urediniospores can attach more easily to the underside of moist leaves vs dry leaves (Nelson, 2009). Additionally, we hypothesized that the infected trees growing in shade would have a higher infection incidence than those growing exposed to the sun, due to the more humid conditions in the shade.

Methods: We searched through 16 municipalities: San Juan, Dorado, Bayamón, Hatillo, Manatí, Trujillo, Las Marías, Mayagüez, Arecibo, Naranjito, Cabo Rojo, Luquillo, Aguadilla, Rincón, Guánica, and Isabela. To locate trees during the pandemic, two different technological approaches were employed: Google Maps and Facebook. During the stay-at-home orders, we used Google Maps Satellite Street View to canvas different neighborhoods and mark potential tree coordinates. After the stay-at-home order were lifted, we visited the marked coordinates and added the trees to my study. Alternatively, we also used Facebook posts to ask people where they had seen *Plumeria*. Once someone indicated a location, we visited it and added the trees to the study. A total of 151 *Plumeria* plants were located using these methods, either wild or cultivated. The following procedure was employed to document them and their ecological factors:

Each *Plumeria* tree species was identified as either *P. rubra, P. alba*, *P. obtusa, P. púdica*, or other (unidentified). If there were any flowers present, it was noted along with their color. The presence or absence of both the leaf rust and the *P. tetrio* caterpillar was documented. The municipality, coordinates, exposure to the sun and elevation of the tree was documented as well. The incidence per branch was determined by selecting a random branch of the tree and counting the number of leaves infected and total number of leaves (both healthy and infected). Infected leaves were collected for further analysis in the lab. Each sample was identified with a letter code identifying the tree and location it came from, in a sequential manner (Example: Sample A1 would be the first sample from location A).

A small portion of an infected leaf tissue was cut, placed in a clean Petri dish with a moist towelette in order to retain moisture. The dish was then left under observation for 10 days. With this, we hoped to promote further sporulation and observe whether there was any additional fungal growth that could indicate the presence of a parasite or another infection, such as *Ramularia coleosporii* reported by (Baiswar *et al.*, 2015).

Data Analysis: The data collected was analyzed using correlations between the prevalence/incidence of the rust and elevation and used ANOVA for the rest of the ecological factors surveyed. A detailed script of the R Studio analyses conducted for both the ANOVA and Correlation statistics can be found below.

Statistical Analysis

1) ANOVA

The analysis of variance is a statistical measure of a variable's significance within a scientific study. It is a way to determine if a set null hypothesis can be rejected or not based on statistical significance. The statistical significance is determined by evaluating whether there are any differences between two or more testing groups (To, 2020). The specific R scripts used for these analyses are included below.

First, the sample set was checked for normality:

```
library(ggplot2)
ggplot(…,aes(x=…, y=…))+
 geom_boxplot()+
geom_point()
ggplot(…,aes(sample=…,color=…))+
geom_qq()+
geom_qq_line()
hist()
qqnorm()
qqline(…, col= "Red")
shapiro.test()
```

Once normalized, the following ANOVA, with pair-wise t-test and Tukey HSD script was performed:

```
name<-aov(… ~ …, data=filename)
class(name)
summary(name)
model.tables(name, type = "means")
pairwise.t.test(…, …, p.adjust.method="none" )
TukeyHSD(name)
```

2) Correlations

For the correlations, the following scripts were used:

```
pairs(data)
plot(data$factor, data$factor)
cor(data)
cor.test(data$factor, data$factor)
```

Aim 2: Determine the genetic diversity of *C. plumeriae* through gene sequencing

Hypothesis: We hypothesized that *C. plumeriae* would show genetic variation in the gene sequenced ITS. The presence of genetic variation in the population could indicate either multiple introductions of the pathogen to Puerto Rico or sexual recombination in the population, but deciding which of these two is the cause was outside the scope of this thesis. Alternatively, if no genetic variation was found in the population, it would suggest a single introduction event and a lack of sexual reproduction.

Methods: The infected leaves collected were scraped with a scalpel to remove the spores. These spores were then placed in 1.5 mL Eppendorf tubes. The scalpel was slightly dulled to avoid cutting into the leaf surface by accident and contaminating the spore sample with plant DNA. The spores then underwent DNA Extraction using the DNeasy Plant Mini Kit. Then these samples underwent Polymerase Chain Reactions (PCR) with the ITS 1 and ITS 4R primers. A more detailed description of the DNA Extraction and PCR procedures can be found below.

DNA Extraction: Phenol-Chloroform Method

The following DNA extraction method was used and adapted from (Quispe-Apaza *et al.*, 2017). Spores were placed in 1.5 mL Eppendorf tubes and macerated in 100 µl DNA extraction buffer 1X for 10 minutes using a small pestle made from sterile pipet tips. A1000 µl tip with the end burned was used to produce a closed, pointed surface. Sterilized fine sand was added to promote cell lysis. The sample was vortexed and the extraction buffer volume was raised to 400 µl. Samples were placed into the heating block set at 65°C for at least 30 minutes to further promote cell lysis. Samples were vortexed regularly throughout heating. After the 30 minutes, samples were placed into a freezer (-18° C) for 10 minutes before being placed again on the heating block for more 10 minutes. Then, 400 µl chloroform: phenol: isoamyl was added, the sample was vortexed and centrifuged at 10,000rpm for 15 minutes.

After centrifugation, the supernatant was transferred to previously labeled new Eppendorf tubes and 30 µl of sodium acetate (5M concentration) was added, as well as 400 µl of isopropanol. Samples were centrifuged for 5 minutes at 10,000 rpm. After centrifuging, the supernatant was transferred to new, labeled tubes and centrifuged again for 5 minutes at the same speed. This centrifugation was repeated twice, until a small pellet was visible at the bottom of the tube. Once the pellet was visible, supernatant was discarded and the pellet was washed twice with 100 µl of 70% ethanol, being careful not to dislodge the pellet from the bottom of the tube. If the pellet was dislodged, then samples were centrifuged again at max speed. Open tubes were placed on a heating block at 35 C for 2 hours to allow the alcohol to evaporate. Alternatively, samples were left to dry at room temperature overnight. Once sufficiently dry, the pellets were resuspended in 100 µl of 1X TE Buffer.

DNA Extraction: DNeasy Plant Mini Kit Method

The following DNA extraction method was adapted from (Quispe-Apaza *et al.*, 2017). Spores were placed in 1.5 mL Eppendorf tubes and macerated in 100 µl of Buffer AP1 for 10 minutes using a small pestle made from sterile pipet tips. A1000 µl tip with the end burned was used to produce a closed, pointed surface. Sterilized fine sand was added to promote cell lysis. The sample was vortexed and the extraction buffer volume was raised to 400 µl. Then, samples were microwaved for 20, 15 and 10 second intervals, with extraction buffer volume being raised as needed. The samples were then placed on the heating block set at 65°C for 45 minutes to further

promote cell lysis. They were vortexed regularly for a few seconds several times throughout heating. After 45 minutes, samples were placed in a freezer (-18° C) for 10 minutes before placing again on the heating block for 10 more minutes.

Once complete, 4 µl of RNase A was added to each sample, vortexed and then incubated for 12 minutes at 65°C. The tubes were inverted 2-3 times throughout incubation. After incubation, 130 µl of Buffer P3 was added. The mixture was mixed and incubated for five minutes on ice. The lysate was centrifuged for 5 minutes a 14,000 rpm and then pipetted into a QIAShredder spin column placed inside a 2 mL collection tube. The sample was centrifuged for 2 minutes at 14,000 rpm. The flow-through was into a new tube without disturbing the pellet (if any). Then, 1.5 volumes of buffer AW1 with ethanol were added and mixed by pipetting. 650 µl of the sample was transferred to a DNeasy Mini Spin column placed in a 2 mL collection tube. They were centrifuged for 1 minute at 8,000 rpm and the flow-through was discarded. This step was repeated for the remaining sample. Next, 500 µl of Buffer AW2 was added to the sample and it was once again centrifuged for 1 minute at 8,000 rpm. The flow-through was discarded, another 500 µl of Buffer AW2 was added and the sample was centrifuged for 2 minutes at 14,000 rpm. The spin column was then transferred to a new 1.5 mL centrifuge tube and 50 µl of Buffer AE was added for elution. The samples were incubated at room temperature for 30 minutes and then centrifuged for 1 minute 8,000 rpm. The last step was repeated for each sample.

Polymerase Chain Reaction (PCR)

In order to prepare samples for PCR, the spore DNA concentration was determined using a Thermo Scientific NanoDrop One. Using these concentrations, sample dilutions were calculated so that the DNA concentrations remained between 30-40 ng/µl. Then, a master mix containing 2 µl of DNA elution buffer, 2 µl $MgCl_2$ (25mg/ml), 0.4 µl dNTPs, 0.2 µl Bio-Rad Taq Buffer, plus 1 µl of each primer (ITS 1 and ITS 4) was added to each tube. Finally, the adequate diluted amount of DNA was added per tube, calculated from the initial concentration of the sample and the total amount of master mix mentioned previously. This resulted in a total of twenty microliters of DNA & PCR primer/master mix solution per PCR tube. The tubes were then placed into a Thermocycler for a period of 2.5 hours, denaturation at 98°C for 1 minute, followed by five cycles of 98°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1:15 minutes. Afterwards, 30 cycles of 98°C for 15 seconds, 53°C for 30 seconds, and 72°C for 1:30 minutes. The final step was one cycle at 72°C for 10 min to final extension. Once the Thermocycler program was finished, a total of 5

microliters,2 µl of loading dye and 3 µl of DNA, per sample were loaded onto a 1% (w/v) agarose gel containing 1X TAE buffer and GelRed™, and electrophoresed for 30 minutes to separate DNA. The PCR samples were cleaned using Exo-CIP™ Rapid PCR Cleanup Kit (New England Biolabs) and sent for sequencing to the UPR Sequencing and Genotyping Facility.

Data Analysis: The sequences were assembled and manually examined for errors using Sequencher 5.4.6 (Sequencher, 2019) and compared with the sequences found in GenBank, to corroborate that our sequence was indeed *C. plumeriae*. The genetic diversity and population structure of *C. plumeriae* was estimated by pairwise population differentiation & F-statistics in R Studio. To estimate if populations were significantly different, 100 replicates of the dataset were generated using the function chao_bootstrap (). Then, summary statistics (mean and 95% CI) were calculated for each of the different parameters of population differentiation. The R-scripts used in this analysis can be found in (Excoffier *et al.*, 1992).

Aim 3.1: Conduct a bioinformatic analysis using a phylogenetic tree for *C. plumeriae* to better understand its evolution

Hypothesis: We hypothesized that *C. plumeriae* from Puerto Rico would be highly related to those reported around different areas of the world.

Methods: The *C. plumeriae* sequences were arranged into a phylogenetic tree, using other *C. plumeriae* sequences from other countries obtained from GenBank, and *Hemeleia vastatrix* (coffee rust) as an outgroup taxon.

Data Analysis: The sequences were prepared using Sequencher 5.4.6 and aligned with the ClustalW software in MEGA X (Kumar *et al.*, 2018). They were compiled into a maximum likelihood phylogenetic tree using RAxML (Randomized Axelerated Maximum Likelihood) version 8.0, explained below (Stamatakis, 2014) and further analyzed using the CIPRES Science Gateway Web server (on RAxML-HPC Black box tool version 8.2.10)(Miller *et al.*, 2010).

RAxML

Randomized Axelerated Maximum Likelihood is a popular program for phylogenetic analysis of large datasets used to arrange them into trees of maximum likelihood. This program

uses the thousands of datasets and lineages available in its database to determine the likelihood of arrangement between species. The algorithm is designed to evaluate the genetic sequences provided and arrange them according its likelihood calculations (Stamatakis, 2014).

Aim 3.2: Conduct a Raman spectra analysis for *C. plumeriae* and *H. vastatrix*

Hypothesis: We hypothesized that the Raman peaks present for *C. plumeriae* from Puerto Rico would be similar to those of *H. vastatrix*, but still distinguishable from each other.

Methods: The spores were analyzed with Raman spectroscopy to determine if any phylogenetic relationships identified in this study were also evident at the morphological level. To do this, spores of both *C. plumeriae* and *H. vastatrix* were collected from some of the evaluated locations. A sample of each was placed on a microscope slide and gently spread in order to separate spore clumps. Six spores from each sample were irradiated individually with a 532nm laser for 60 seconds on a Thermo Scientific DXR Raman Spectrometer at the UPR Molecular Sciences Research Center in order to obtain the Raman spectra. The spectra were downloaded as a CSV file for analysis.

Data Analysis: The resulting Raman spectra were compiled, averaged, normalized and visualized using Microsoft Excel. The same procedure was repeated for each sample and they were compared by species and by location.

Sources of Information

The sources of information for this work include live plant tissues of *Plumeria spp.*, fungal spores of *C. plumeriae.* We obtained four PCR samples of purified and positively amplified DNA for sequencing. No humans or animals were part of this study, and the samples collected from the trees did not harm its ability to continue to grow since we were only removing already infected tissues.

List of Materials, Equipment & Software

Materials

1) QIAGEN DNeasy Plant Mini Kit, 69104
2) Fisher Brand 1.5 mL Eppendorf tubes
3) Fisher Brand 1000 µl sterile pipet tips
4) DNA Extraction Buffer 2X (2% CTAB, 1.4 M NaCl, 100mM Tris, 20mM EDTA, 2% PVP, 0.4% Mercaptoetanol)
5) Sodium acetate (25 mg/ml concentration)
6) 70% Ethanol, Sigma-Aldrich, 459844-4L
7) 1X TAE Buffer
8) ITS 1 Primer
9) ITS 4 Primer
10) Agarose, Fisher Scientific, BP160-100
11) Milli-Q Purified Water
12) Glass microscope slides
13) $MgCl_2$ (25mg/ml concentration), Promega, A351H
14) 0.4 µl dNTPs, (10mM concentration), Invitrogen, 18427-013
15) 0.2 µl Bio-Rad Taq Buffer, M0480L
16) Exo-CIP™ Rapid PCR Cleanup Kit (New England Biolabs)
17) GelRed™ Invitrogen S33102

Equipment

1) Thermo Scientific NanoDrop One
2) Gilson Electronic Pipettes
3) Heating block (Dry Bath Incubator), Fisher Scientific
4) Freezer (-18° C)
5) Sprout Mini Centrifuge
6) Eppendorf Centrifuge, 5415D
7) Genie 2 Vortex, Fisher Scientific
8) Fume Hood
9) Bio-Rad MJ Mini Thermocycler

10) Thermo Scientific Owl Easy Cast B2 Gel Electrophoresis System
11) Thermo Scientific DXR Raman Spectrometer at the UPR Molecular Sciences Research Center

Software

1) R Studio v1.3
2) OMNIC Raman Microscopy Software
3) Microsoft Excel 2016
4) Sequencher version 5.4.6 (Sequencher, 2019)
5) MEGA X (Kumar *et al.*, 2018)
6) RAxML (Randomized Axelerated Maximum Likelihood) version 8.0 (Stamatakis, 2014)
7) CIPRES Science Gateway Web server (on RAxML-HPC Black box tool version 8.2.10) (Miller *et al.*, 2010)

Section 3: Results, Discussion, & Conclusions

Aim 1: Evaluate the prevalence and incidence of *C. plumeriae* on *Plumeria spp.* in Puerto Rico and determine the impact of ecological factors on both prevalence and incidence.

After canvassing sixteen municipalities, we located a total of 151 Plumeria trees. Of these trees, 70 were identified as *Plumeria rubra*, 49 as *Plumeria alba* and 13 as *Plumeria obtusa*. The trees not successfully identified (a total of 18) were classified as "other". An Excel sheet containing the full list of trees identified for this study can be found in **Appendix A**. of these151 *Plumeria* trees, 90 trees were not infected with *C. plumeriae* at the time of identification, while 61 trees were infected. Therefore, the calculated prevalence for *C. plumeriae* in our survey was 40.4%. A summary of these results and their relation to geographic location in Puerto Rico can be found in **Figure 12**.

***Figure 12**: Plumeria tree canvassed locations and relation to C. plumeriae infection.* The figure demonstrates that only three of the sixteen canvassed municipalities were found to have only healthy *Plumeria* trees. These municipalities (marked blue) were Rincón, Trujillo Alto and Arecibo.

The incidence and ecological factors for each of the trees was documented and can also be found in the Appendix A. To determine if there were any significant differences among the disease prevalence and incidence, statistical analyses were conducted in R Studio. The first ecological factor that was evaluated was host species. **Figure 13** shows the breakdown of disease incidence per host species. From this figure, it is apparent that the *Plumeria rubra* species was the most commonly infected species found during this survey, with the highest incidence values. Yet,

Plumeria obtusa had the highest average incidence. This corresponds with the literature which suggests that *P. rubra* and *P. obtusa* are both highly susceptible to *C. plumeriae* infection (Nelson, 2009). However, after conducting ANOVA statistical analysis on both the disease incidence and prevalence, we found that disease prevalence was significantly different across host species ($F(4,146) = 12.53$, $p = 8.69e\text{-}09$*). Disease prevalence did not differ significantly ($F(3,55) = 1.52$, $p = 0.22$). A full summary of the ANOVA results for host species can be found in **Table 2**.

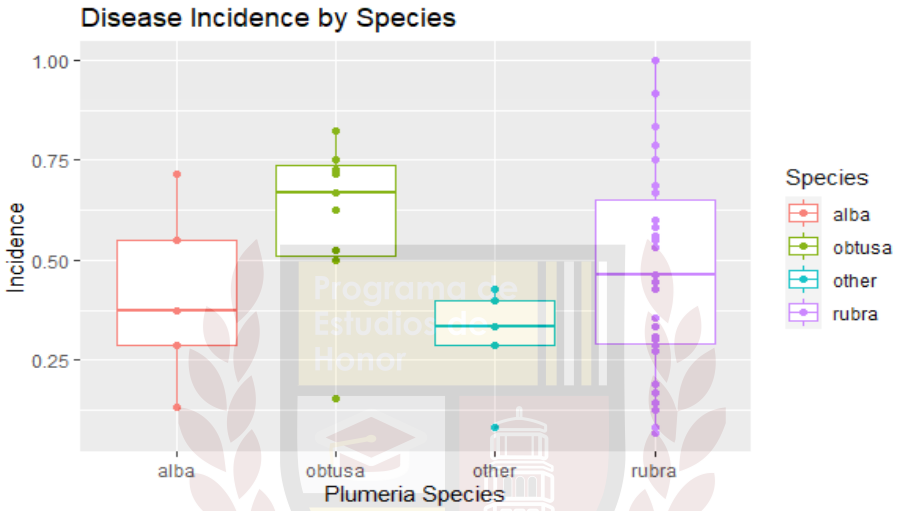


***Figure 13**: Disease Incidence by Host Species.* This boxplot demonstrates the distribution of disease incidence according to the host species. The Plumeria rubra species had the highest number of infected trees in the survey, as well as the highest percentage of incidence within a single branch.

The next ecological factor evaluated in the study was the exposure to sunlight. This category was split into three dimensions: fully exposed to sunlight, partially exposed to sunlight and completely in shade. In **Figure 14** we can see the average distribution of incidence per exposure to sunlight. The highest number of infected trees were found to be growing fully exposed to the sun. However, the highest incidence percentages were recorded among those only partially exposed to sunlight. According to the ANOVA tests, incidence was not significantly different across any of the categories. Meanwhile, disease prevalence was shown to be significantly different between categories ($F(2,148) = 15.56$, $p = 7.36e\text{-}07$*). Next, the presence of herbivores was evaluated for both disease incidence and prevalence. However, neither categories yielded significant results. This led us to believe that herbivores might not be a disease vector as previously thought. The results of these ANOVA tests can also be found in **Table 2**.

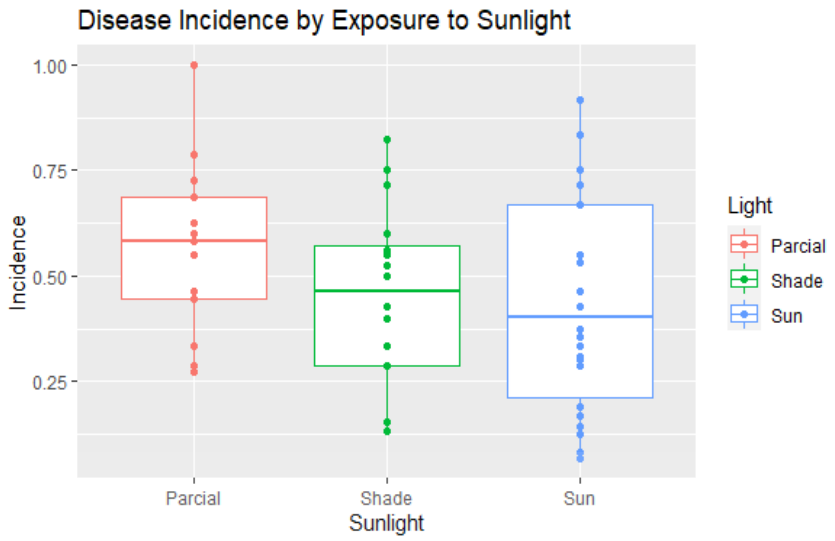


***Figure 14**: Disease Incidence by Exposure to Sunlight.* All three categories had varying degrees of disease incidence. The partially exposed to sunlight had the highest incidence percentages. Meanwhile, those fully exposed to sunlight had the highest frequency of incidence.

***Table 2**: Results of ANOVA Statistics for Ecological Factors*

ANOVA & Tukey Outcomes of Ecological Conditions Impact on Incidence & Prevalence			
Category	**Prevalence (n=151)**	**Incidence (n=61)**	**Significant Variances**
Sunlight	F (2,148) = 15.56 p = 7.36e-07**	F (2,56) = 1.60 p = 0.212	Sun-Parcial (p= 0.0000172) Sun-Shade (p= 0.0005639)
Species	F (4,146) = 12.53 p = 8.69e-09**	F (3,55) = 1.52 p = 0.22	obtusa-alba (p=0.0000002) rubra-alba (p=0.000008) other-obtusa (p=0.00019) rubra-obtusa (p=0.02859)
Herbivores	F (1,149) =0.003 p = 0.96	F (1,57) = 0.064 p = 0.80	none

The next factor evaluated was that of elevation. Since elevation is a quantitative variable, correlational tests were conducted to determine if there was a significant difference across prevalence or incidence. A summary of the statistical results can be found in **Table 3**. Disease

prevalence was found to be significantly and positively correlated to elevation (p = 0.002, R = 0.25).

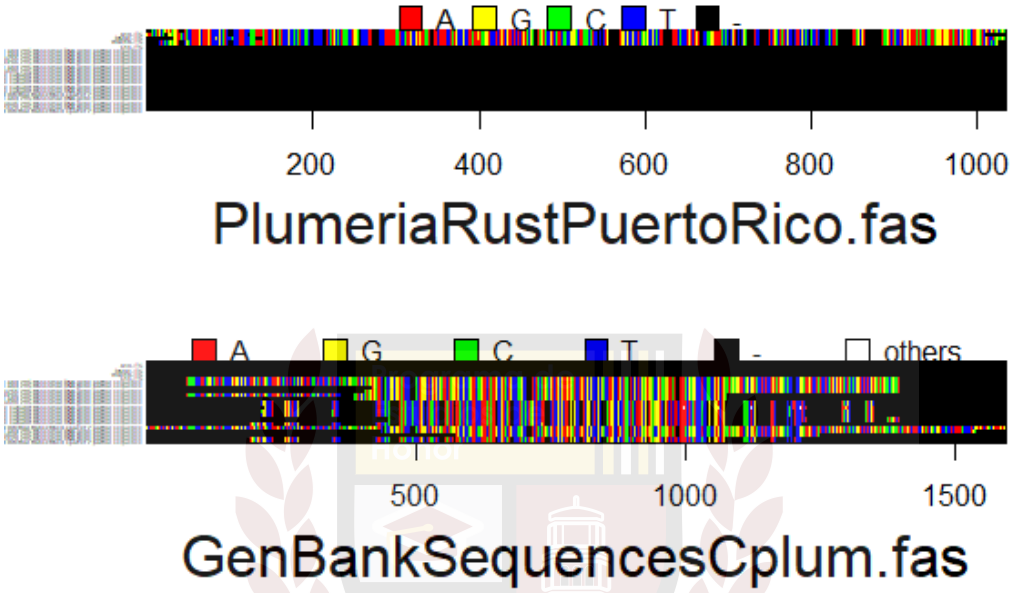
***Table 3**: Results of Correlation Statistics for Elevation and Disease*

Correlations of Elevation and Disease Prevalence vs Elevation and Disease Incidence			
Category	**Sample Size (n)**	**p value**	**R**
Elevation and Prevalence	151	0.002**	0.25
Elevation and Incidence	61	0.17	0.18

Aim 2: Determine the genetic diversity of *C. plumeriae* through gene sequencing

To determine genetic diversity, the sequences obtained from the UPR Sequencing and Genotyping Facility were combined with other *C. plumeriae* sequences obtained from the GenBank and analyzed in R Studio. These statistics showed that there was no significant difference between the local *C. plumeriae* population and the ones reported in the GenBank. **Table 4** summarizes the results of the statistic tests conducted.

***Table 4**: Summary of Pairwise Statistics for Genetic Diversity and Overview*

Significance Test for Pairwise Nei's Gst Statistics		
		
Locus	**Mean**	**95% CI**
Plumeria Rust Puerto Rico	0.3750	(0.096-0.654)
GenBank Sequences	0.1953	(0.065-0.326)
Global Estimate based on average heterozygosity	0.2446	(0.114-0.375)

Aim 3.1: Conduct a bioinformatic analysis using a phylogenetic tree for *C. plumeriae* to better understand its evolution

The phylogenetic tree for *C. plumeriae* was compiled and calculated as described in the methodology and can be found in **Figure 15**. The tree demonstrates strong bootstrap values (100) grouping between the *Coleosporium* species. All of the *Coleosporium plumeriae* species are grouped together in the tree, further indicating that though these strains are from different parts of the world, they are still more similar to each other than to other *Coleosporium* species. The phylogenetic tree shows that *C. plumeriae* of Puerto Rico are more closely related to *C. plumeriae* of India and Indonesia. However, the Puerto Rico samples also appear to be highly variable among each other. Yet, the low bootstraps values (<70) in those branches of the phylogenetic tree imply that a sample is either too diverse, too similar or too ambiguous to be related confidently.

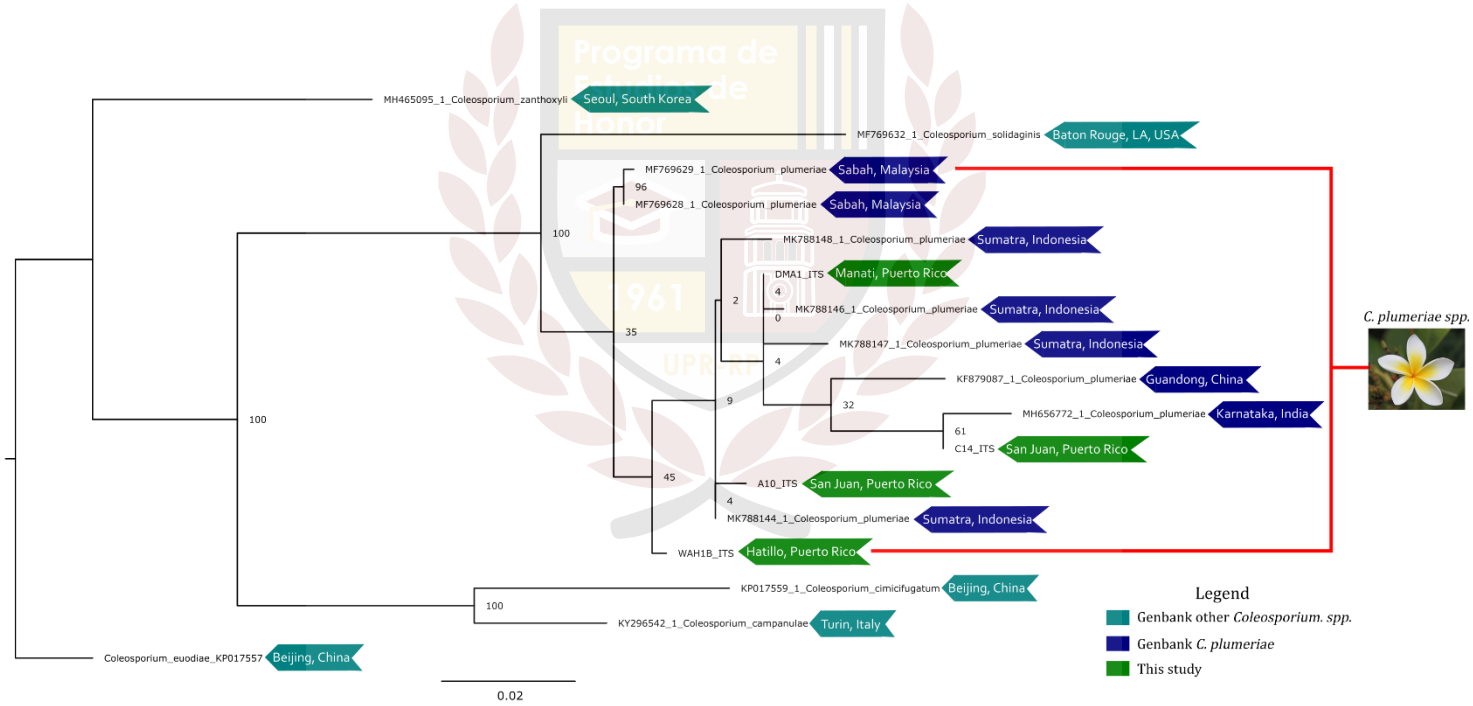


Figure 15: *Maximum Likelihood Phylogenetic Tree for Coleosporium plumeriae*. Numbers at nodes show bootstrap values. Species of individuals per clade and source is shown at the right. Sources of additional sequences: *C. euodiae*, KP017557; *C. campanulae*, KY296542, (Garibaldi *et al.*, 2017); *C. cimifugatum*, KP017559; *C. plumeriae*, MK788144, MK788147, MK788146, MK788148 (Oliveira *et al.*, 2019); MH656772; KF879087, (Yang *et al.*, 2014); MF769628, MF769629 (McTaggart & Aime, 2018); *C. solidaginis*, MF769632, *C. zanthoxyli*, MH465095 (McTaggart & Aime, 2018); The tree was rooted to the outgroup *Coleosporium euodiae*, KP017557.

Aim 3.2: Raman spectra analysis for *C. plumeriae* and *H. vastatrix*

The results of the Raman spectroscopy analysis were divided between the *C. plumeriae* and *H. vastatrix* species. Samples for both species demonstrated relative uniformity between samples and minimal distinctions between the two leaf rusts. **Figure 16-A** contains the Raman spectra for *C. plumeriae*. The spectra show two significant peaks at approximately 950 and 1,250 Raman shifts, which correspond with potential aromatic bonds or polyethylene C-C bonds. There are also smaller peaks at the 2,250 and 3,000 range which correspond with C≡C bonds and CH or OH bonds, respectively. Though all the samples display homogeneity within each sample, there were variations in the band intensities between samples from different locations. Overall, all *C. plumeriae* samples had the same significant peaks.

The Raman spectra for *Hemileia vastatrix* is shown in **Figure 16-B**. The spectra for this species show significant peaks at 1,000 and 1,500 Raman shifts, which correspond with C-C bonds and potential aromatic bonds respectively. Most of the samples also showed smaller peaks at approximately 2,250 and 2,400 which could indicate the presence of C≡C bonds and another small peak at approximately 2,750-2,850 which could indicate an ethane (CH_2) bond. Overall, the Raman spectra for these two species, while similar, are still pretty distinguishable from each other.

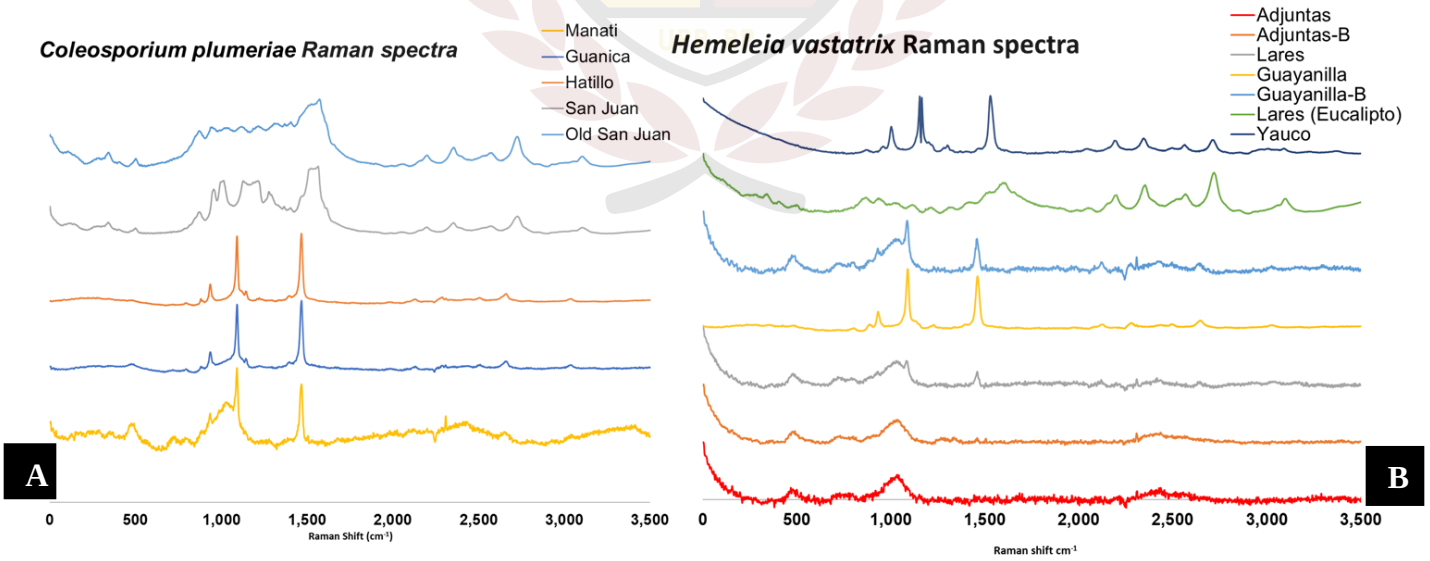


Figure 16: *Raman Spectra for C. plumeriae and H. vastatrix*

Conclusion

The results for the first aim suggest that *C. plumeriae* is highly prevalent in Puerto Rico. *C. plumeriae* prevalence was 40.4%, which indicates a high prevalence as we initially hypothesized. The results of the statistical analyses further suggest that *C. plumeriae* prevalence is significantly linked to exposure to sunlight, elevation and host species, but not to the presence of herbivores. However, *C. plumeriae* incidence was not significantly linked to any of the ecological factors. Therefore, our hypothesis that *C. plumeriae* distribution would vary due to environmental conditions among the *Plumeria* spp. on the island and that those growing in shade would have a higher infection incidence was partially supported.

The results for the second aim suggest that the Puerto Rico populations of *C. plumeriae* are relatively similar to their GenBank counterparts around the world. There was no significant difference between these two populations which would potentially support our hypothesis that expected to find relatively low genetic diversity for *C. plumeriae*. However, when combined with the results for the third aim, another possibility emerges. According to our phylogenetic tree, the *C. plumeriae* sequences from Puerto Rico and the GenBank are 100% related to each other when compared to other *Coleosporium* species (Bootstrap values: 100). Yet, the Puerto Rico strains display some high variability between each other which was unexpected. This suggests that the genetic diversity *within* the Puerto Rico population itself may be more variable than its relationship to the GenBank sequences seems to suggest. Additionally, the high relatedness of the Puerto Rico strains to the GenBank samples from Asia suggest there was possibly multiple introductions of the fungus into the island through imported trees from Asia. This would begin to shed some light on our second aim hypothesis which expected to find some more variability in the genetic diversity due to multiple introductions. Nonetheless, the low bootstrap values observed for the Puerto Rico strains make the results too ambiguous to be able to draw a formal conclusion. Additionally, the results of this study also reflect the limitations due to the lack of data available for *C. plumeriae* samples from Latin America and North America in the GenBank. Our third aim hypothesis is nevertheless supported, since the two *C. plumeriae* populations appear to be highly related. Additionally, though the Puerto Rico *C. plumeriae* spores were highly variable phylogenetically, they were relatively similar to each other at the Raman spectra level, yet distinguishable from other rust species (*H. vastatrix*).

Future Directions

Future work could look to increase the sampling size, particularly targeting trees from the southeastern part of the island. More sequences from Puerto Rico using the ITS gene could be obtained to be able to draw more solid conclusions regarding the genetic diversity of this fungus. Other variable genes, such as the intergenic spacer 1 (IGS1), could be amplified and sequenced as well (Niazmand *et al.*, 2013). Finally, an additional pathogenicity study could be conducted to more accurately determine the susceptibility of different *Plumeria* species to *C. plumeriae*.

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