Integration of Rapid Ohia Death Research through Forest Pathology and Pathogen Genetics

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We now have a much better understanding of rapid ohia death (ROD) than we did when this proposal was submitted for HISC funding in 2016. Funding was requested for work to be conducted in or coordinated from Iowa, including genetic and other analyses on strategically collected isolates of *Ceratocystis*. These and other collaborative efforts would provide tools for defining the diseases caused by *Ceratocystis* sp. A and sp. B, their mechanisms of dispersal, geographic distributions, projected pathways, and management strategies. These goals were to be addressed through fulfillment of four technical objectives.

After a summary of the problem as stated in 2016, highlights of accomplishments and on-going studies are listed in bullet form. The fulfillment of the four technical objectives are then presented. The implications of these findings are placed in the context of the overall research progress by the ROD research team. Principal research cooperators in Hawaii included Lisa Keith, Wade Heller and Marc Hughes (USDA-ARS, PBARC), JB Friday (UH), Flint Hughes (USFS-IPIF), Carter Atkinson and Kyle Roy (USGS), and Ryan Perroy (UH Hilo).

First year's funding was provided by HISC and DOFAW. The genomic DNA sequencing of the *Ceratocystis* species and their relatives was to be conducted by two collaborators, but Kealoha Kinney (USFS-IPIF) and Matt Templeton (Plant and Food Research, NZ) were unable to provide these valuable and expensive data. In a change of plans, the critical genomic sequencing and analyses were conducted at Iowa State University with a second year's funding from DOFAW. This report includes the second year's genetic work, whose analyses are still in progress.

PROJECT SUMMARY FROM 2016

At the time of the proposal, more than 30,000 acres of rapid ohia death (ROD) had been recorded, but the specific causes of the mortality had not been clearly identified. Two undescribed species of *Ceratocystis* (sp. A and B) were associated with ROD. Productive collaborations and a forest pathology approach had resulted in considerable progress in the 6 mo previous to the proposal. The proposed genetic studies were designed to fill in critical gaps and provide tools for a solid understanding of ROD.

I proposed that there were at least three distinct diseases that had been referred to as ROD: 1) Ceratocystis wilt, the most alarming and potentially explosive disease, caused by *Ceratocystis* sp. A, 2) ohia crown dieback, a less important disease caused by *Ceratocystis* sp. B, and 3) an unidentified disease in the Kilauea area of Volcano National Park associated with discolored wood in the lower stem, consistent with a root disease. Revisiting the Kilauea area in subsequent trips led me to conclude that the last mortality is caused by a death of ohia roots due to an episodic abiotic agent, perhaps geothermal activity, and it is not addressed further in this report.

HIGHLIGHTS OF ACCOMPLISHMENTS

1. Description of the new species *C. lukuohia* and *C. huliohia*, and the diseases they cause, were supported in part by data and observations from this project. The Hawaiian taro pathogen, *C. uchidae,* was also described as new.
2. Genetic studies suggested that only the Latin American *C. lukuohia* should be able to cause the dramatic wilt disease ravaging ohia populations, and inoculation studies confirmed that *C. lukuohia* should be the focus of management.
3. Mating studies showed the potential for new strains to be generated through interspecific crosses: *C. lukuohia* was able to sexually cross with the Hawaiian *Syngonium* strains and *C. platani*, while *C. huliohia* was able to cross with the Hawaiian *C. uchidae*.
4. Draft nuclear genomes were generated through sequencing at Iowa State University for 15 isolates of *C. lukuohia*, 3 isolates of *C. huliohia*, and 15 isolates of related *Ceratocystis* species.
5. The mitochondria genomes of the 33 isolates were fully annotated, and a survey with new DNA markers showed strong evidence for movement of group II introns and gene coding sequences from *C. huliohia* into many of the 67 surveyed isolates of *C. lukuohia*.
6. A new qPCR protocol that is ~100 times more sensitive than the currently deployed protocol was developed for detection of the two pathogens, allowing for quantification of minute amounts of *C. lukuohia* and *C. huliohia* in air samples and soil.
7. Rotorod sampling technology for airborne DNA of the *Ceratocystis* species was developed and deployed across Hawaii Island, confirming that the pathogens are airborne miles downwind from inoculum sources, thus supporting current management practices.
8. Through exhaustive comparisons of the 15 nuclear genomes of *C. lukuohia*, 21 polymorphic microsatellite markers were developed that can be used for DNA fingerprinting of isolates and characterization of *C. lukuohia* populations.
9. An initial database of the microsatellite alleles for the 21 loci has been developed with 91 isolates of *C. lukuohia* (and 12 isolates of *C. huliohia*) from across Hawaii Island. The database can be later expanded to trace the origins of new outbreaks in future decades.
10. The analyses of the 91 isolates demonstrated a center of diversity at the origin of the epidemic in Lower Puna and a second population around Hilo, as well as possible spread of *C. lukuohia* southwest with prevailing winds and north with storm tracks (Fig. 1).

ON-GOING AND PROPOSED STUDIES (WITH PBARC SCIENTISTS)

1. More rigorously determine if *C. lukuohia* and *C. huliohia* can cross sexually and exchange nuclear DNA.
2. Determine if vegetative structures of *C. lukuohia* and *C. huliohia* can fuse, exchange cytoplasm, and introduce highly mobile genetic elements to the other species.
3. More fully analyze the microsatellite data to test hypotheses concerning origin and movement of *C. lukuohia* populations.
4. Expand the microsatellite database of *C. lukuohia* with additional isolates (e.g., Waimea and Kalopa St. Park) in order to trace the origins of new disease foci.
5. Apply the microsatellite markers to DNA extractions from positive rotorod samples collected at remote sites in order to genotype/trace the source of the *Ceratocystis* DNA.

6. Develop a better genome assembly and annotation with long sequence reads of the ex-type culture of *C. lukuohia* through cooperation with PBARC scientists.
7. Examine pathogenicity determinants with cooperators in Brazil by comparisons to the sequenced and annotated *C. cacaofunesta* genome.
8. Determine the extent of introgression of *C. huliohia* genes into the 15 sequenced nuclear genomes of *C. lukuohia*.
9. Develop a set of polymorphic microsatellite markers for *C. huliohia* that can be used for genotyping and population characterization (e.g., Kauai vs. Hawaii Island populations).

TECHNICAL ACCOMPLISHMENTS – BY OBJECTIVE

OBJ. 1 - WE WILL GENETICALLY CHARACTERIZE HAWAIIAN ISOLATES OF CERATOCYSTIS, focusing on DNA sequences and microsatellite markers for comparisons with more than 1000 worldwide isolates of the Ceratocystis fimbriata *complex. Isolations from select locations, trees and symptomatic tissue will allow characterizations of the two new diseases, their disease cycles, dispersal mechanisms, population structure and biogeography (with Keith Lab). Historical imagery may help identify the initial foci of the diseases (Teresa Menard,TNC).*

In a series of trips from early 2016 through early 2018, I isolated one or more *Ceratocystis* species from 94 ohia trees on Hawaii Island. I visited and obtained isolates from *Syngonium* at two nurseries in Hilo, apparently the only nurseries in the region that have *Ceratocystis* (personal communication, Lisa Keith). Isolates of *C. fimbriata* were obtained from locally-grown sweet potato storage roots sold in the Hilo market. Two isolates of a *Ceratocystis* sp. on taro corms on Oahu and Kauai were obtained from Janice Uchida (UH). At a minimum, rDNA sequencing was conducted on each isolate, and further sequencing of the mating type genes were conducted on a subset of these isolates for phylogenetic analyses.

The DNA sequences identified 82 of the ohia isolates as *Ceratocystis* sp. A (now *C. lukuohia*, Barnes et al. 2018), and these isolates were found to be most closely related to *Ceratocystis* populations native to the Caribbean and Central America. The closest relatives of *C. lukuohia* were the cause of canker stain of planetree (*C. platani*), an undescribed species from coffee in Costa Rica, and the *Syngonium* and *Xanthosoma* strains of the *C. fimbriata* complex (including *Syngonium* isolates from Hilo nurseries). The coffee pathogen from Colombia (*C. colombiana*) and rubbertree strains from Guatemala and Mexico were somewhat more distant relatives of *C. lukuohia* by DNA sequence analysis. The sweet potato pathogen (*C. fimbriata* in the strictest sense) was more distantly related, in the South American subclade of the *C. fimbriata* complex, along with *C. cacaofunesta* and Brazilian strains of *C. fimbriata*.

Twelve of the 94 ohia isolates were identified by DNA sequences analysis as *Ceratocystis* sp. B (which has since been described as *C. huliohia*, Barnes et al. 2018), and phylogenetic analyses showed that it is very closely related to the taro fungus from Oahu and Kauai (which we named *C. uchidae*, Li et al. 2018) and the Asian species *C. changhui* and *C. cercfabiensis.*

Based on the known biology of its nearest relatives (Caribbean subclade members of the Latin American Clade), *C. lukuohia* would be expected to be capable of causing a vascular wilt disease. As expected, it was the only *Ceratocystis* isolated from rapidly dying ohia trees that had

deep, radial penetration of the host sapwood. This rapid wilting and systemic colonization were confirmed in a preliminary inoculation study (with Marc Hughes) of mature ohia trees, in which the fungus quickly moved up the sapwood of inoculated trees at up to 10 cm per day, causing wilt symptoms in a few months. The Asian relatives of *C. huliohia* are not aggressive pathogens, and inoculation of ohia trees showed limited movement in the host and production of canker-like symptoms.

The initial set of 14 microsatellite markers that we have used extensively for *Ceratocystis* species showed a remarkable lack of variation in an initial set of 64 isolates of *C. lukuohia*; a single unique allele was found in one isolate from Leilani Estates. This is the purported epicenter of the epidemic, and 2009 imagery (from T. Menard) documented that ohia trees had already been dying at that time. Thus, this Lower Puna area may have the oldest populations of *C. lukuohia* and would most likely have accumulated the most mutations and have the greatest genetic diversity. The *C. huliohia* isolates also lacked variability, and their microsatellite alleles were most similar to those of its Asian cousins, *C. uchidae*, *C. cercfabiensis*, and *C. changhui*.

The near lack of variability in both of the ohia pathogens suggested that each likely originated from a single genotype that had been introduced relatively recently, perhaps in the last two decades. More variable markers for *C. lukuohia* were developed based on genome sequencing at Iowa State University (Objective 3), and these new markers were used for a more detailed population study (Objective 4).

OBJ. 2 - WE WILL IDENTIFY THE POTENTIAL FOR STRAINS TO MATE AND PRODUCE NEW AGGRESSIVE PLANT PATHOGENS by developing tester strains and conducting crosses in the lab. Isolates collected in a survey of Hawaiian nurseries and ohia isolates will be the focus.

Mating studies in *Ceratocystis* require development of special tester strains of the two opposite mating types. In our published pairing studies (Barnes et al. 2018), the tester strains of *C. lukuohia* mated readily with *C. lukuohia* testers of the opposite mating type. Reduced fertility (fewer fruiting bodies, fewer spores, and some aborted spores) were seen in pairings with tester strains of *C. platani*, *C. colombiana*, and *Syngonium* isolates from Hawaii and Florida. Although these interspecific pairings produced fewer progeny, some progeny were viable, and there were recombinant phenotypes among the progeny from these hybrid crossings. There was a very low level of interfertility with tester strains of *C. lukuohia* and *C. cacaofunesta*, and no interfertility with a Hawaiian isolate of *C. fimbriata* from sweet potato.

Tester strains of *C. huliohia* of opposite mating type were fully interfertile with each other and partially interfertile with tester strains of *C. uchidae* and *C. cercfabiensis*, another Asian species, but the *C. huliohia* strains were not interfertile with *C. polychroma* testers. Further pairings with special cycloheximide tester strains are being conducted to see if a sexual cross between *C. lukuohia* and *C. huliohia* is possible under forced conditions, but it is highly unlikely. However, genome analyses suggest that these two species may be able to exchange mitochondria DNA through vegetative interactions, and nuclear DNA may be similarly exchanged through vegetative interactions. Such vegetative exchanges of DNA between *C. huliohia* and *C. lukuohia* are currently being investigated in the lab.

OBJ. 3 - WE WILL SEQUENCE THE GENOMES OF HAWAIIAN CERATOCYSTIS *SPP. AND RELATIVES in a collaborative study coordinated through ISU. The initial sequencing and genomic analyses of 25 isolates (Templeton, Kinney) will be supplemented with ISU sequencing and analyses. These data will be used to understand the origins of the ohia pathogens (Bennett, Kinney), how they colonize and kill ohia trees (Keith), and how they interact with insects, microbes and biocontrol agents (Bennett, Kinney).*

Unfortunately, the collaborators at IPIF and in New Zealand were unable to provide the proposed genome sequencing, and this sequencing had to be carried out at Iowa State University, with the financial support of DOFAW. In total, Illumina sequencing was carried out at Iowa State University for 15 isolates of *C. lukuohia*, three of *C. huliohia*, and 15 isolates of their nearest relatives. Initial assemblies of the nuclear and mitochondria genomes of each of the 33 isolates have been made, with thorough annotations of the mitochondria genomes recently completed.

The mitochondria genomes of all the *Ceratocystis* species are very large, and variable in size among isolates of *C. lukuohia*, primarily due to an abundance of highly mobile group II introns. Comparisons of the mitochondria genomes provided very strong evidence of recent genetic exchange between *C. huliohia* and *C. lukuohia*, wherein some isolates of *C. lukuohia* have Asian group II introns from *C. huliohia* that were likely horizontally transferred by hyphal fusions (vegetative interactions) in recent years. PCR based markers of 12 of the mobile introns of *C. huliohia* were developed and have been tested against 67 isolates of *C. lukuohia*. Each of the 12 introns are in all isolates of *C. huliohia* and in some but not all isolates of *C. lukuohia*, mostly in isolates from sites where both species are coinfecting the same trees.

A more thorough nuclear genome assembly of the ex-type isolate of *C. lukuohia* is now available through hybrid assembly of the Illumina reads (short reads) with PacBio sequences (long reads). We are also planning to use other long-read sequences generated at PBARC by Nanopore technology (Scott Geib, USDA-ARS) in order to have an improved nuclear genome assembly. It is hoped that this more thorough assembly will be annotated by colleagues in Brazil, and it will be used to improve assemblies of the other *C. lukuohia* isolates. These assembled genomes will be studied for genes that may explain the exceptional aggressiveness of *C. lukuohia* to ohia trees. Lastly, the introgression of nuclear genes from *C. huliohia* into the genome of *C. lukuohia* can be determined if we are able to continue with this work in spring 2019.

OBJ. 4 - WE WILL DEVELOP NEW MOLECULAR MARKERS FOR POPULATION AND DISPERSAL STUDIES from the genome analyzes in obj. 3. Highly polymorphic markers will identify the relatedness of populations and identify the origins of new foci (see obj. 1). Spore samplers from Iowa will be tested in Hawaii, and diagnostic, multi-copy gene targets will allow for detection of extremely low levels of airborne spores (Bennett, Atkinson).

Improved detection of C. lukuohia *and* C. huliohia — We developed new qPCR protocols to detect extremely low levels of DNA of the two respective pathogens, *C. lukuohia* and *C. huliohia*. Initially, we collaborated with colleagues in Italy who developed a similar qPCR protocol for the closely related *C. platani* (Lumia et al. 2018), and then we adapted the protocol for the Hawaiian species. The qPCR tests use the internal transcribed spacer region 1 of the

tandemly repeated rDNA operon, and the new protocol is about 100 times more sensitive than the diagnostic ceratoplatanin qPCR protocols. Although such sensitivity is not needed for the routine diagnoses conducted at PBARC, the new technology allows detections of minute amounts of *Ceratocystis* DNA in the air or in soil. Field crews have also used special forensic DNA sampling wands to collect minute amounts of ambrosia beetle frass, and the qPCR protocol has been able to detect *C. lukuohia*.

Sampling airborne inoculum — Rotorod samplers were brought to Hawaii to determine if the new qPCR protocols could detect windborne inoculum (ambrosia beetle frass) of the *Ceratocystis* spp. Working with Wade Heller, Eva Brill and Lisa Keith (PBARC), the new qPCR assay, rotorod sampling, and DNA extraction protocols were optimized for detection of airborne DNA. These protocols have been successfully used since January 2018 across the Big Island to detect the two species, which are each detected sporadically near, and downwind from, sources of inoculum. These data are being compiled by Wade Heller and are not incorporated into this report.

Microsatellite fingerprinting and population genetics — Twenty-one new microsatellite markers were developed by exhaustive comparisons of the 15 nuclear genomes of *C. lukuohia*. These novel markers are surprisingly polymorphic and were able to detect specific series of new mutations that have arisen from the originally introduced genotype (Fig. 1). Analysis of the 82isolates that I collected and 21 isolates from the PBARC lab show the greatest accumulation of mutant alleles in Lower Puna, the expected origin of the epidemic. Isolates that have only the most common alleles are displayed as dark red circles in the figure, and this is considered the original genotype. Somewhat surprisingly, isolates from Kona show the fewest accumulation of mutant alleles. Pink circles represent isolates that had only one or two rare alleles unique to one site or isolate. Dark purple squares and dark green triangles represent the earliest mutations (Fig. 1), with lighter squares and triangles representing isolates that have those mutations plus later accumulated mutations, in-series.

Local populations apparently derived from southwest movements of the fungus with prevailing winds were genetically diverse and comprised upwind genotypes. The sampled isolates from the scattered mortality from Waiakea to Kapapala showed substantial diversity, and some of this mortality is associated with wounding, especially by animals.

The light green triangles represent a geographically restricted Hilo population centered from Stainback Highway to the old canefields west of Hilo, where these closely related genotypes may have arisen. Google Earth imagery showed a developing outbreak of Ceratocystis wilt as early as 2012 in remnant ohia forests at the western edge of the old canefields, but no isolates are available from the 2012/2013 outbreak.

In contrast to the scattered mortality over space and time, many incidences of concentrated outbreaks have been identified around the island, where many trees appear to be simultaneously infected through exposure to strong wind events. At each of these episodic events, the isolates from the location showed surprising genetic uniformity based on microsatellite markers. This genetic uniformity indicates that the trees were infected by inoculum from a very limited source, perhaps from a single tree or group of trees. One of the Hilo genotypes was isolated from 10

trees in an outbreak that began in 2015, after Tropical Storm Iselle. Three of the trees were at the north end of the old canefields and seven trees were at the Lower Wailuku management site at Pi'ihonua, where many trees were felled to reduce inoculum. Two isolates from the remote outbreak near Pahoehoe Stream collected in 2017 (two light green triangles northwest of Hilo in Fig. 1) appeared to be derived from the Hilo genotypes, but they had some distinct alleles, suggesting that they had a similar but distinct inoculum source compared to the Pi'ihonua outbreak that began in 2015.

Isolates from the 2015/2016 outbreaks at both Kamae'e Stream and Waipunalei on the Hamakua Coast (light purple squares) were genetically uniform, but the two populations are distinct from each other and appear to be separately derived from Puna genotypes. The jump north over the "Hilo genotypes" (light green triangles) suggests that the Kamae'e Stream and Waipunalei genotypes were brought northward by a storm event like Tropical Storm Iselle.

The 2017 Kohala episode resulted in a population that is genetically uniform and unique (blue upside-down triangles) and distinct from the Kona, Kamae'e and Waipunalei populations, showing that these were not the sources of inoculum for the critical Kohala outbreak. However, it should be pointed out that only a small subsample of the large Kamae'e population has been sampled, and a nearby group of trees could have been the source of inoculum for the Kohala outbreak. The Kohala population shares some genetic characteristics of the Pahoehoe Stream and Hilo area populations, but the origin of this outbreak does not appear to be any of the populations that have been sampled thus far.

Lastly, the 2015 Upper Wailuku outbreak north of Saddle Road affected trees along a stand edge with exposure to west winds, perhaps an unusual Kona wind event. Surprisingly, the single genotype found there was most similar to the genotype found in the 2016 Kona outbreak, where mortality appeared suddenly from Telephone Exchange Rd. in South Kona to Holualoa (red circles in Fig. 1). The seven Kona isolates were genetically uniform, with the exception of a single allele in one isolate, but new isolates from further south in Kona have not yet been fingerprinted.

Microsatellite database — The microsatellite database should prove an invaluable asset in future monitoring of the epidemic in the decades to come. The database and protocol are designed to be handed off to a new lab so that the database can be expanded as new outbreaks are discovered. In the near term, the PBARC team intends to generate comparable data using a different technique, which will allow for validation of the microsatellite database. For future study, the microsatellite database will be more easily maintained and updated than the genome sequencing approach that may be taken at PBARC.

IMPLICATIONS AND FORTHCOMING WORK

The genetic analyses were proposed to fill critical knowledge gaps and provide tools for further research. Accomplishment of the objectives has contributed substantially to our much better understanding of the cause of rapid ohia death. In 2016, two species of *Ceratocystis* were both thought responsible for the massive loss of ohia trees on the Big Island. Phylogenetic studies, a more critical association of the pathogens with symptomatology, and inoculation studies strongly

suggest that only the species from the Caribbean subclade of *Ceratocystis* is capable of causing the devastating wilt-type disease. The other *Ceratocystis* species was closely aligned with weakly pathogenic species in Asia and was found to cause only cankers in inoculations. Although it is frequently detected in dead and dying ohia, including on Kauai, the Asian species does not appear to be substantially contributing to the outbreaks seen on Hawaii, and management should focus on Ceratocystis wilt.

The single most important research contribution to our understanding of ROD has been the descriptions of *Ceratocystis* sp. A and B as *C. lukuohia* and *C. huliohia*, respectively, and the diseases they cause (Barnes et al. 2018). The descriptions were justified, in part, by phylogenetic analyses conducted in Iowa and elsewhere, but the publication included important distinctions in symptomatology of the two diseases. Mating tests with the ohia pathogens and their nearest relatives were conducted in Iowa, and these were also included to help justify designation of the two new species names.

GENOMIC STUDIES AND GENETIC PLASTICITY

The *Syngonium* strain in Hilo nurseries and the taro strain in Hawaii (now recognized as *C. uchidae*) were shown to be very closely related to the respective ohia pathogens based on genetic analyses and mating studies. Somewhat surprisingly, we were able to successfully cross the *Syngonium* and taro strains, respectively, with *C. lukuohia* and *C. huliohia* in the laboratory, though there was a reduction in the number of viable progenies in the interspecific crossings. Thus, these other strains or newly introduced strains of *Ceratocystis* could potentially lead to new recombinant strains of the ohia pathogens that could be more aggressive on ohia or other hosts. However, the genomic analyses showed that the respective ohia pathogens did not arise directly from the *Syngonium* or taro strains, in contrast to what had hypothesized 2016.

The most surprising finding is that *C. lukuohia* has incorporated mitochondria DNA from *C. huliohia*. If we can get a full assembly of the nuclear genome and have it annotated by our colleagues in Brazil, then we should be able to determine if there also has been exchange of nuclear DNA between the two species. Scott Geib at PBARC may be doing more long-read sequencing of *C. lukuohia* that can be incorporated with our data to produce much better assemblies of the nuclear genome. If we are able to continue this work, introgression analyses should identify any movement of nuclear genes from one species to the other.

We are currently conducting laboratory studies to see if *C. lukuohia* and *C. huliohia* can exchange DNA through sexual crossings, but it is much more likely that vegetative structures of the two species can fuse and transmit mobile DNA. We are hoping to test the possibility of vegetative transmission of DNA between the species and determine if the transmission of mitochondria DNA from *C. huliohia* happened just once in the early history of the epidemic or if the two species still genetically interact when co-infecting a tree. This may have important implications for predicting if new interspecific recombinants of *C. lukuohia* are possible, if *C. lukuohia* is genetically fixed and cannot change appreciably, or if the *C. lukuohia* population may deteriorate by acquisition of parasitic DNA from *C. huliohia*.

POPULATION DYNAMICS AND MANAGEMENT

The precise pathways that the two ohia pathogens took into Hawaii are not clear, but they each appear to have arisen from a single genotype or spore based on the genomic analyses and the microsatellite data. Importation of live plants was the likely culprit. The identified center of *C. lukuohia* microsatellite diversity was in the Lower Puna, the likely source of diversification, if not the original introduction of the pathogen. This is consistent with the Pictometry imagery that showed dying ohia trees near Leilani Estates in 2009, before homeowners recognized the mortality.

More important for management and predicting the spread of the epidemic is the further support for the hypothesis that the pathogens are airborne and *C. lukuohia* is moving north, most likely in ambrosia beetle frass. The data suggest movement of *C. lukuohia* out of Puna, both as the gradual northerly expansion of new genotypes acquiring new alleles and in the apparent jumps of "light Purple" Puna genotypes to Kamae'e Stream and Waipunalei (Fig. 1).

There has been extensive cutting at Waipunalei to reduce infectious ambrosia beetle frass, and the cutting may have temporarily generated airborne inoculum, while the Kamae'e site has not been well studied or managed. Isolates from newly infected trees around these areas and northward should be genotyped to see if the Kamae'e or Waipunalei genotypes have spread. The Kohala outbreak is also associated with essentially a single genotype. Although it cannot be traced to any particular source, the Kohala population is now fingerprinted and could be compared to new outbreaks on Hawaii or Maui, should *C. lukuohia* spread to there.

Microsatellite analyses found a differentiated population in and around Hilo. It is suspected that these genotypes arose from the old canefields west of Hilo, where the disease was apparently present in 2012. The Lower Wailuku management site at Pi'ihonua involved felling of many trees, but the Pi'ihonua genotype has not been found elsewhere, and we have no evidence that the felling of infected trees leads to new outbreaks of Ceratocystis wilt. The two Pahoehoe Stream isolates appear to be from the Hilo population, but they comprise two unique genotypes. More isolates should be obtained from the Middle Wailuku area to see if Hilo genotypes dominate there (though two sampled trees there were infected with Puna genotypes). The Middle Wailuku area has many hundreds of diseased trees, and this area could be a source of inoculum for the Hamakua or Kohala areas with the right storm.

The Ka'u population is very similar genetically to the population in Lower Puna, and prevailing winds may have brought infectious inoculum directly from Puna to Ka'u. Further mortality in Kona should be studied for microsatellite alleles to see if the Kona genotype is spreading or if new genotypes are coming into the region, especially from Ka'u.

In the last 2 years, a slow and steady increase in mortality has been noted from the Mountain View/Fern Forest area to Bird Park in Volcano National Park and Kapapala. Some of this mortality has been associated with animal damage. The genotypes identified in this area match closely those of upwind populations from Waiakea to Puna. Thus, much of the current mortality in the Kilauea area could be associated with inoculum carried in the prevailing winds, perhaps unassociated with major wind events. Rotorod sampling has detected *C. lukuohia* over a mile

downwind from an inoculum source in Volcano National Park. In such regions with remote and diffuse sources of inoculum, the most effective management might be to avoid wounding and use a fungicidal wound dressing on fresh wounds.

The microsatellite database is designed to be easily built upon by others, and it is hoped that it will be used by scientists at PBARC in the coming decades. It would be possible to develop similar microsatellite markers for *C. huliohia* by using genome sequences and the pipeline we developed designing the *C. lukuohia* microsatellite markers. The *C. huliohia* markers would be particularly helpful in comparing the Kauai and Hawaii populations of *C. huliohia*, as well as other populations of *C. huliohia* that are likely to be discovered on other islands.

Development of the new qPCR protocol allows for detection of small amounts of fungal DNA, less than that found in a single spore. This detection protocol and the rotorod sampling techniques developed from this study are being deployed by Wade Heller and others across the island. These studies and other studies support the hypothesis that *C. lukuohia* and *C. huliohia* are principally disseminated in the wind in the form of fungal spores in liberated ambrosia beetle frass. The rotorod sampling thus far indicates that DNA of these fungi can be sporadically abundant at sites downwind from ROD trees. There is the opportunity to expand the microsatellite genotyping to the DNA extracted from the rotorod samples to better trace the source of airborne inoculum. This work should prove valuable in evaluating management strategies and prioritizing areas for treatment.

In summary, the four proposed objectives were fulfilled, and much more was accomplished than was initially anticipated. A much better understanding of the *Ceratocystis* species in Hawaii and their diseases is now known, and new tools are available to further research, monitoring and evaluation of management strategies. The new qPCR protocols and rotorod sampling are working well, and the preliminary data support the hypothesis that the *Ceratocystis* species are airborne and that management should focus on reducing airborne inoculum in ambrosia beetle frass. Mating studies and other genetic analyses point to the dangers of further interactions of *C. lukuohia* with *C. huliohia* and other introduced Hawaiian *Ceratocystis* species. The basic genomic studies are complex and ongoing, but initial analyses have provided valuable microsatellite tools and a database for tracing the source of outbreaks and predicting further spread of *C. lukuohia* populations. Extending these genetic studies should reap more valuable information and applicable tools.

PUBLICATIONS RELEVANT TO THE PROJECT

Barnes, I., A. Fourie, M. J. Wingfield, T. C. Harrington, D. L. McNew, L. S. Sugiyama, B. C. Luiz, W. P. Heller, and L. M. Keith. 2018. New *Ceratocystis* species associated with rapid death of *Metrosideros polymorpha* in Hawaii. Persoonia – Molec. Phylog. Evolut. Fungi 40:154-181. https://doi.org/10.3767/persoonia.2018.40.07

Li, Qian, T. C. Harrington, D. McNew, and J. Li. 2017. *Ceratocystis uchidae* sp. nov., a new species on Araceae in Hawaii and Fiji. Mycoscience 58:398-412.

Lumia V., V. Modesti, A. Brunetti, C. L. Wilkinson, G. Di Lernia, T. C. Harrington and M. Pilotti. 2018. Real-Time PCR for *Ceratocystis platani* detection: in-depth validation to assess the diagnostic potential and include additional technical options. iForest, 11:499-509.

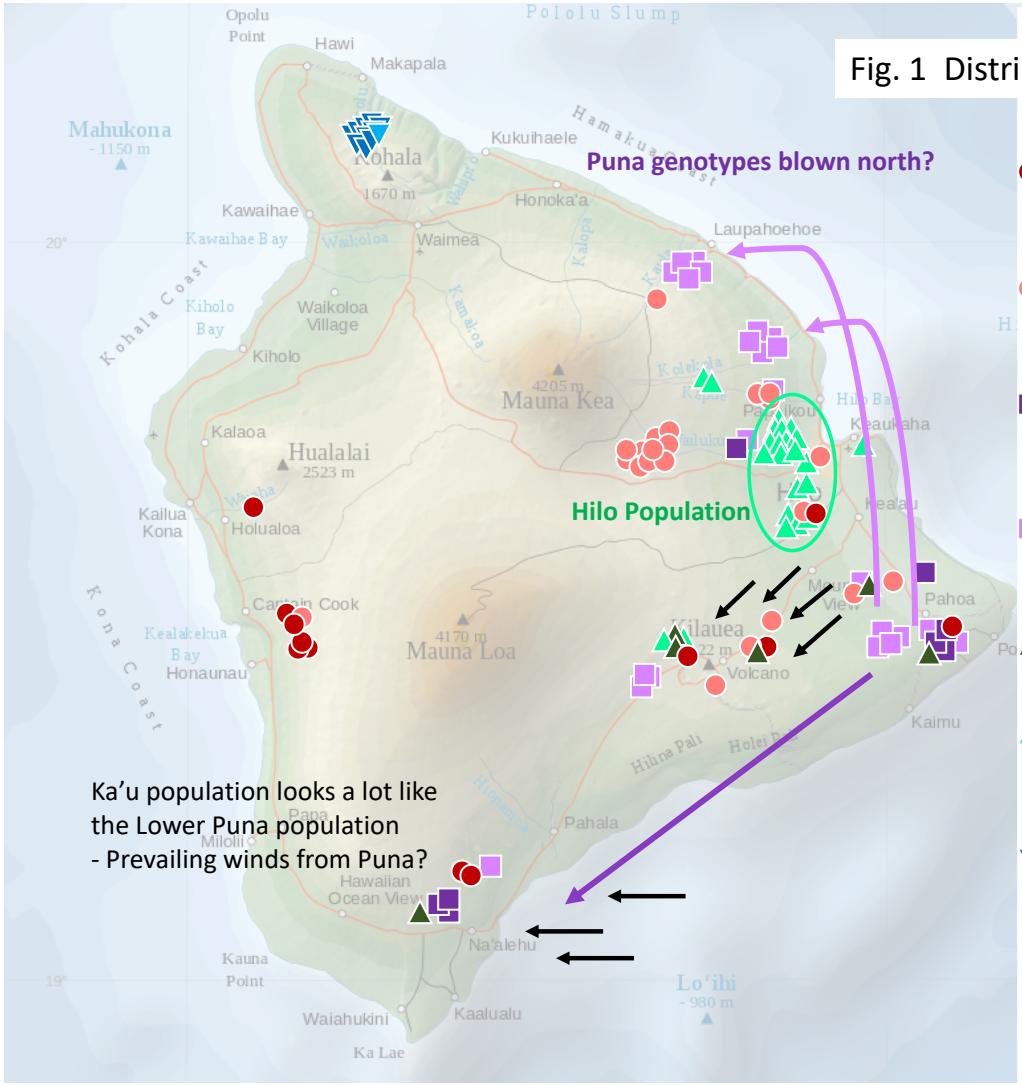

Fig. 1 Distribution of microsatellite genotypes of *Ceratocystis lukuohia*

- Only the most common allele at each of the 21 loci (the most common and perhaps the original genotype, no accumulated mutations)
- Same as red, except for one or two unique alleles peculiar to a site (different genotypes independently derived from the red genotype)
- Differing from the red genotype by a single mutant allele that is dominant in Lower Puna (perhaps a mutation that developed early in the epidemic)
- Many different genotypes derived from the dark purple genotype (mostly Lower Puna, but Kamae'e and Waipunalei outbreaks, too)
- A rare genotype, differing from the red genotype by single mutant allele (in Puna, Volcano and Ka'u)
- Many different genotypes derived from the dark green genotype, most encountered in the Stainback Rd. to Pi'ihonua areas (from old canefields west of Hilo)?
- Kohala outbreak represented by 11 isolates, a distinct genotype, but most similar to green triangle genotypes (Pahoehoe Stream and Hilo area).