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## Research

# High-Sensitivity ITS Real-Time PCR Assays for Detection of *Ceratocystis lukuohia* and *Ceratocystis huliohia* in Soil and Air Samples

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**Abstract**

Rapid Ohia Death (ROD) is caused by two pathogens, *Ceratocystis lukuohia* and *Ceratocystis huliohia*. The established species-specific real-time PCR assays targeting the single-copy cerato-platanin gene require DNA from between 2 and 16 spores per reaction for consistent detection, which is suitable for analysis of infected plant tissue but not sensitive enough to consistently detect low spore loads in environmental samples. Here, we present two redesigned qPCR assays targeting the first internal transcribed spacer (ITS) region of the multi-copy ribosomal DNA operon from the respective species, both capable of consistent detection of the pathogen at concentrations as low as 1 fg per reaction, less than the size of the haploid genome. Due to this increased sensitivity, these ITS qPCR assays are superior for analysis of DNA extracted from material collected in airborne particle samplers and from soil. Here, we demonstrate the utility of these qPCR assays for the characterization of windblown and soilborne dispersal of the pathogens, which is critical for the development of management practices that mitigate disease spread.

*Keywords*: dispersal, frass, soil, spore

Rapid Ohia Death (ROD) is caused by the fungal pathogens *Ceratocystis lukuohia* and *Ceratocystis huliohia* (Barnes et al. 2018; Hughes et al. 2020), both of which are known only to exist within the Hawaiian Islands. Both species were discovered by isolation of the fungi from killed ohia lehua (*Metrosideros polymorpha*) trees and were originally detected in the Puna and South Hilo districts of Hawaii Island (Keith et al. 2015; Mortenson et al. 2016). Ohia lehua is the predominant native tree in Hawaii's native forests, with critical roles in forest ecology and watershed management, and it is highly valued for cultural reasons. Extensive monitoring and detection efforts, including the use of spectral imaging from aircraft (Vaughn et al. 2018), as well as high-resolution imaging from helicopters and drones (Perroy et al. 2021) to spot symptomatic trees, have aided in the identification of new disease outbreak areas on Hawaii Island. Confirmation of the presence of either pathogen has relied on real-time PCR assays targeting the single-copy cerato-plantin gene from each species (Heller and Keith 2018), and these assays have been demonstrated to be highly specific for their target species. By

2019, both species had been detected on Kauai (Brill et al. 2019; Heller et al. 2019), and *C. huliohia* was detected on Maui and Oahu (*unpublished results*). The ecological threat of the spread of the ROD pathogens, particularly for the more virulent of the two species, *C. lukuohia*, has been modeled based on favorable environmental growth conditions for the pathogen (Luiz and Keith 2021), the presence of *M. polymorpha* landcover, and test results of samples collected from the abovementioned efforts (Fortini et al. 2019).

Anthropogenic involvement in the arrival of the exotic ROD pathogens to Hawaii has been hypothesized, possibly involving the shipment of infected plant material or contaminated solid wood packing materials in shipping containers (Barnes et al. 2018). Additionally, there has been a major public outreach campaign to "stop the spread" of ROD, the main messages from which have been to prevent the movement of infected wood, avoid injuring healthy ohia trees, and sanitize gear, tools, and vehicles before and after entering the forest (Roy et al. 2020b). Interisland quarantine restrictions have been in place since 2016, limiting the movement of ohia plant material (including wood), as well as soil from islands with confirmed detections of *C. lukuohia* or *C. huliohia* (Hawaii Administrative Rules 2016). Subsequently, importation regulations prohibiting the movement of all *Myrtaceae* were established to prevent the spread of fungal diseases (Hawaii Administrative Rules 2020).

In addition to spread through movement of infected plant material, airborne dispersal of the ROD pathogens has been implicated as a major mechanism of spread, especially through spores contained in the frass of wood-boring beetles (mainly *Xyleborus* spp. and *Xyleborinus* spp.), which are known to colonize infected trees (Hughes et al. 2022; Roy et al. 2019, 2020a). Airborne spread of ambrosia beetle frass from killed trees has been thought to be an important means of spread of other *Ceratocystis* species, especially species in the Latin American clade (Harrington 2013; Iton 1960), but there has been only limited experimental evidence to support windborne dispersal of *Ceratocystis* species (Luchi et al. 2013). Short- and long-term weather patterns (e.g., prevailing wind direction or wind speed during tropical storms) are postulated to impact how far away and where such spore-laden frass is deposited; furthermore, susceptible wounds are required for spores to establish an infection court in the xylem of a healthy tree (Hughes et al. 2020). Land use practices, such as ranching or conservation management, which enclose or exclude ungulate animals known to strip, scrape, and debark ohia trees, have also been shown to influence the incidence of ROD-associated mortality at multiple sites across Hawaii Island (Perroy et al. 2021).

Attempts to quantify the airborne inoculum load using commercially produced or low-cost air samplers (Atkinson and Roy 2023; Atkinson et al. 2019) have been marginally successful in confirming the presence of DNA of both *Ceratocystis* spp. in windblown particles, although there have been few positive samples, and the sensitivity of the detection method (i.e., ceratoplatanin qPCR) has come into question. A different real-time PCR assay targeting the second internal transcribed spacer (ITS) of *C. platani* has been used for detection of fungal DNA in airborne inoculum traps (Luchi et al. 2013), so it stands to reason that qPCR targeting the ITS locus of other *Ceratocystis* spp. might also be advantageous for detecting airborne spread. To improve the sensitivity of detection for surveillance and detection of these pathogens from environmental DNA samples, we present here an additional set of qPCR assays targeting the first ITS (rDNA ITS1) of the multi-copy ribosomal DNA operon as a modification of an existing method developed for *C. platani* detection (Pilotti et al. 2012). We expected that such a sensitive detection method would allow for testing the hypothesis that the *Ceratocystis* species are dispersed in airborne particles and in soil.

## MATERIALS AND METHODS

### Primer and probe design

Primer pairs and probes within the rDNA ITS1 region were adapted from the *C. platani* ITS assay published by Pilotti et al. (2012) and validated by Lumia et al. (2018). The ITS sequences of the two ohia pathogens and their relatives, including *C. platani*, were aligned with the Pilotti et al. qPCR oligonucleotides. Probes and primer pairs for *C. lukuohia* and *C. huliohia* were designed to overlap with the corresponding aligned nucleotide positions of the published assay, and individual nucleotides were changed to their respective target species' sequence (Table 1). Primers and PrimeTime (5′6-FAM, ZEN/Iowa Black FQ) probes for ITS assays were synthesized by Integrated DNA Technologies (Coralville, IA). Placement of the ZEN internal quencher was determined with the company's design tool; the complete probe sequences with the 6-FAM fluorophore and quenchers are 5′-/56-FAM/CGGTRCCCT/ZEN/TCAGAAGGGCCCTACCAC/3IABkFQ/-3′ for C.luku.ITS.Pr and 5′-/56-FAM/AAAACCTTA/ZEN/TAGAAGGGGCCCCCCAACTAC/3IABkFQ/-3′ for C.huli.ITS.Pr.

### Real-time PCR

Preliminary primer/probe combinations were initially tested for efficiency, repeatability, and cross-reactivity with a BIO-RAD Multicolor iQ5 Real-Time PCR Detection System (Hercules, CA). All real-time PCR data reported here were generated using an Applied Biosystems QuantStudio 5 instrument (Thermo Fisher, Waltham, MA) and SensiFAST Probe reaction mix (Meridian Bioscience, Cincinnati, OH), with results consistent with the BIO-RAD instrument. Reactions (20 µl total volume) contained 10 µl of the reaction mix, 500 nM primer, 200 nM probe, and up to 5 µl of extracted DNA. As an internal control, the *C. lukuohia* and *C. huliohia* ITS assays were routinely multiplexed with the MeNu47 qPCR assay (Heller and Keith 2018) to amplify spiked-in *M. polymorpha* DNA (10 pg/reaction) using the previously reported 400 nM primer and 100 nM probe concentrations. Control reactions containing only the spike-in *M. polymorpha* DNA were included in each run for Ct value comparison. The thermocycling protocol consisted of a 2-min initial denaturation at 95°C, followed by 50 cycles of 10 s at 95°C and 30 s at 60°C. Duplicate reactions for each multiplex, *C. lukuohia*/MeNu47 and *C. huliohia*/MeNu47, were run for each environmental detection sample, and triplicate reactions were run for the standard curve experiment. Selected qPCR products were cloned into vector pCR4-TOPO (Thermo Fisher) according to the manufacturer's protocol and submitted to Eurofins Genomics (Louisville, KY) for Sanger sequencing using primers M13F and M13R to confirm that the detected fragment was that of the targeted *Ceratocystis* species. Performance of the *C. lukuohia* and *C. huliohia* ITS qPCR assays was also evaluated and confirmed to function as expected on StepOne Plus (Thermo Fisher) and CFX Touch (BIO-RAD) instruments (data not shown).

### Sample collection and DNA extraction

Rotorod samplers (Model 20, IQVIA, Parsippany, NJ) were used for collecting samples of wind-dispersed fungal DNA. Samplers were mounted 1.5 m off the ground and powered by 12 V DC batteries and solar panels (Fig. 1). Sampling sites were selected

within ohia stands confirmed to be infested with *C. lukuohia* and *C. huliohia* and in widely dispersed open (wind-exposed) sites across Hawaii Island (Fig. 2), as well as on Maui and Molokai. Sampling duration ranged from 72 to 96 h (twice weekly collections). Retractable sampling heads were cleaned (20-min soak in 10% bleach, followed by autoclaving at 121°C for 15 min) after use, then reloaded with new plastic rods, coated with silicone grease (IQVIA), and placed into zip top bags for transport back to the field. Samples (plastic rods coated with debris stuck to silicone grease) were placed into NucleoSpin Forensic filters (Macherey-Nagel, Bethlehem, PA) along with 100 mg of 0.8-mm ceramic grinding beads and the recommended volume of extraction buffer from the NucleoMag DNA Forensic kit (Macherey-Nagel). Filter tubes were heated at 65°C for 10 min prior to 60 s of homogenization at maximum speed (FastPrep 24, MP Biomedicals, Solon, OH). Tubes were centrifuged for 1 min at 10,000 × *g*; then, the clarified lysate was transferred to a deep well block for purification following the kit manufacturer's protocol on a KingFisher Flex instrument (Thermo Fisher). Negative controls (unexposed plastic rods) were included to verify the absence of lab contamination.

Concentrated *C. lukuohia* and *C. huliohia* DNA was purified from isolates P17-58 and P17-59 (USDA-ARS-PBARC collection) using a commercial spin-column extract kit (NucleoSpin Plant II Kit, Macherey-Nagel) and quantified using a Qubit 2.0 fluorimeter (Thermo Fisher). For standard curves, a 10-fold serial dilution series containing 100 ng to 1 fg per reaction was prepared for each sample. For environmental detection samples, *M. polymorpha* DNA for the MeNu47 spike-in control was isolated from leaves using the same spin-column extraction kit and added to the reaction master mixes at 10 pg per reaction.

Soil samples were received from various partners and consisted of soil collected from forests or boot brush sanitation stations at the indicated geographic locations. Extracts from 250 mg of soil material were prepared using a commercial spin column kit for soil (DNeasy PowerLyzer PowerSoil Kit, Qiagen, Germantown, MD) following the manufacturer's recommended protocol, except that samples were homogenized (FastPrep-24) at maximum speed for 1 min. For some samples, fungal isolations were conducted with the same samples using a carrot baiting method (Moller and Devay 1968) with modifications. Carrots were surface sterilized by soaking for 10 min in 10% bleach (0.6% sodium hypochlorite final concentration), then peeled and cut into slices ~1 cm thick. Soil baiting was performed by placing several carrot slices into zip top bags containing approximately 250 ml of soil and observing for fungal structures (mycelium and perithecia) after 2 to 4 weeks of room-temperature incubation. Unsuccessful attempts to isolate *Ceratocystis* spp. from plastic rotorods involved sandwiching the rods between two carrot slices wrapped in Parafilm.

## RESULTS AND DISCUSSION

The amplicon lengths of the qPCR products are 98 bp for *C. lukuohia* and 110 bp for *C. huliohia*. *Ceratocystis lukuohia* is closely related to *C. platani*, and only 2 to 4 nucleotide (nt) changes per oligo of the Pilotti assay (2012) were needed to match the *C. lukuohia* sequence. In contrast, all but 7 nt at the 3′ end of the reverse primer and 8 to 14 nt (30 to 46% of the total positions) of the *C. huliohia* forward primer and probe were changed to match the new target. To account for intraspecies variation and enable equivalent hybridization of the *C. lukuohia* probe against both the ITS-A and ITS-C sequence variants identified by Heller and Keith (2018), one position was synthesized with a nucleotide mixture (R = A/G, underlined nucleotide in Table 1) to provide equal specificity. The indels distinguishing the Hawaii Island and Kauai *C. huliohia* populations (Heller et al. 2019) are outside of the qPCR amplicon described here.

Cross-reactivity of the *C. lukuohia* and *C. huliohia* ITS qPCR assays was observed when using very high concentrations of purified fungal DNA, such as in the standard curve experiment, which utilized a mean of three replicates with nearly identical results. Cross-reaction of the *C. lukuohia* ITS assay on *C. huliohia* DNA occurred with ≥1 ng total input DNA, whereas *C. huliohia* detection of *C. lukuohia* DNA only occurred at ≥100 ng input, the highest level tested (Fig. 3). The calculated PCR efficiencies based on the slopes of the standard curves are 101.5% for *C. lukuohia* and 101.0% for *C. huliohia*, nearly ideal values, validating the reproducibility and repeatability of the assays. In all cases, a ΔCt of >15 cycles was noted between the Ct value for the genuine target relative to the cross-reaction. A panel of 24 DNA extracts previously used to validate RPA assays for *C. lukuohia* and *C. huliohia* (Atkinson et al. 2017), which contains three additional *Ceratocystis* spp. as well as an *Ophiostoma* sp. and *Leptographium bistatum*, was used to further investigate the cross-reactivity of the new assays (Supplementary Table S1). Similarly low (high Ct value) cross-reactivity was noted for the *C. lukuohia* assay on concentrated DNA from *C. fimbriata* isolated from *Ipomoea batatas* and *C. uchidae* from *Colocasia esculenta* and for the *C. huliohia* assay on concentrated DNA from *C. fimbriata* from *Syngonium podophyllum*. Reactivity with low Ct values in the range of 13 to 19 cycles was as expected for the *C. lukuo-*

**TABLE 1**

ITS qPCR primer and probe design for *Ceratocystis lukuohia* and *C. huliohia* based on the primer and probes for *C. platani*

| Target | Oligo name | Sequence (5′-3′) | Tm (°C) | Reference |
|---|---|---|---|---|
| *C. platani* | C.P.Sn.For.I | CGTACCTATCTTGTAGTGAGATGAATGC | 63.9 | Pilotti et al. 2012 |
| *C. lukuohia* | C.luku.ITS.For | **A**C**A**TACCTATCTTGTAGTGAGATGAAT**TG**[a] | 62.3 | This report |
| *C. huliohia* | C.huli.ITS.For | **CG**T**AAA**CTATCTTGT**GAA**GAGATGAA**C** | 61.3 | This report |
| *C. platani* | C.P.Sn.Rev.I | GAGTTTACAGTGGCGAGACT---ATACTG[b] | 63.4 | Pilotti et al. 2012 |
| *C. lukuohia* | C.luku.ITS.Rev | GTTTACAGTGGCGAGACT**TAT**ATACTG | 62.3 | This report |
| *C. huliohia* | C.huli.ITS.Rev | **TTTTAG**T**GGT**G**AAGAAGATTACT**TATACTG | 61.4 | This report |
| *C. platani* | C.P.TM.Pr. | CGGTGCCCTTCAGAAGGGCCCTACCACC | 74.6 | Pilotti et al. 2012 |
| *C. lukuohia* | C.luku.ITS.Pr | CGGT**R**CCCTTCAGAAGGGCCCTACCAC[c] | 71.5–73.6 | This report |
| *C. huliohia* | C.huli.ITS.Pr | **AAAAC**C**TTAT**AGAAGGG**G**CC**CC**CCA**AC**T**AC** | 69.8 | This report |

[a] Boldface indicates change relative to *C. platani* assay.
[b] Dash (–) indicates gap in sequence alignment.
[c] Underline indicates a mixed base code.

*hia* assay on DNA from *C. fimbriata* from *S. podophyllum* and for the *C. huliohia* assay on DNA from *C. uchidae* due to the close evolutionary relationships of these species pairs and their nearly identical ITS sequences (Barnes et al. 2018). Importantly, these additional *Ceratocystis* spp. on *I. batatas*, *C. esculenta*, and *S. podophyllum* affect soft tissues, mainly on storage roots or vines in greenhouses, and they are not airborne or expected to be present in environmental samples. Cross-reactivity of the Pilotti *C. platani* ITS qPCR assay was also noted by Lumia et al. (2018) when they added ≥50 pg of fungal DNA from various nontarget *Ceratocystis* species from the Latin American, North American, African, and Asian-Australasian clades. For additional positive confirmation of genuine amplification of *C. lukuohia* or *C. huliohia* ITS sequences, such as unexpected detections in a new geographic area, qPCR products can be sequenced to confirm they match the expected species. As discussed below, the PCR products from 16 soil samples were cloned and Sanger sequenced, and each of the sequences matched those of *C. lukuohia* or *C. huliohia* and not the other *Ceratocystis* species.

**FIGURE 1**
Rotorod sampler setup. Includes Model 20 Rotorod Sampler, rods pointing downward, mounted 1.5 m off the ground on PVC pipe, which also supports a 12V solar panel connected to a battery; stakes and three guywires support the setup.

Although it is important to be mindful of cross-reactivity, the amount of template fungal DNA required for cross-reactivity, ≥1 ng for the *C. huliohia* assay and ≥100 ng for the *C. lukuohia* assay, far exceeded the expected concentration of target fungal DNA in environmental samples. The tradeoff for analytical sensitivity over analytical specificity is necessary due to the low concentration of target DNA in environmental samples, but it does not negatively impact the fitness of the ITS qPCR assays in terms of their diagnostic specificity for their intended purpose to detect and quantify DNA of *C. lukuohia* or *C. huliohia* from low-concentration sources. DNA extracts from highly concentrated sources, such as from cultured isolates or colonized plant tissue, should not be tested for diagnostic purposes with this method and are better suited for the cerato-platanin qPCR assays (Heller and Keith 2018). In contrast, environmental samples including soil, swabs, and air samples often have such low concentrations of target DNA that they are below the sensitivity limit of the cerato-platanin assays and are better suited for the ITS assays that have no issue of cross-reactivity in this range. As an example of the contrast in sensitivity for environmental sampling, the cerato-platanin assays rarely detected *C. lukuohia* and *C. huliohia* in air samples on Hawaii Island if the samplers were greater than 250 m from the nearest infestation (Atkinson and Roy 2023), yet we routinely detected these species in air samples using the new assays.

Based on a recently completed *C. lukuohia* CBS 142792 nuclear genome sequence assembly (GenBank ASM2350984v1), the haploid genome size is 30.6 Mbp, equivalent to 31.4 fg DNA


**FIGURE 2**
Map of Rotorod sampler locations. Circles indicate sampler locations used 1/19/2018 to 5/10/2018; squares indicate locations used 5/14/2018 to 1/28/2019. Triangles indicate summits of Hawaii Island's five volcanoes. Numbers correspond to the location descriptions in Table 2. Circles with dashed and dotted outlines indicate geographic groupings of samples near known confirmed trees infected with *Ceratocystis lukuohia* and/or *C. huliohia*. Shaded relief map provided by Forest and Kim Starr.

using the conversion factor of 978 Mbp per pg (Dolezel et al. 2003), with 80 to 100 copies of the rDNA operon per haploid genome. Remarkably, we have confirmed detection of the ITS qPCR targets with as low as 1 fg fungal genomic DNA input per reaction (Fig. 3), equivalent to just 3.18% of the genomic DNA content of a single spore. This level of sensitivity is slightly higher than the reported minimum 2 and 3 fg sensitivities of the Luchi and Pilotti assays for *C. platani*, respectively (Luchi et al. 2013; Lumia et al. 2018; Pilotti et al. 2012).

Using our new assays, we found that 39 and 17% of 580 airborne particle samples were positive for *C. lukuohia* and *C. huliohia* DNA, respectively (Table 2). Detections were made at Hawaii locations nearby stands or forests affected by both pathogens, as well as at distal sites tens of kilometers from the nearest confirmed infected trees, such as the collectors placed within Pohakuloa Training Area (PTA) at high elevation in the saddle between Mauna Kea and Mauna Loa (#22 and 23; PTA sites in Fig. 2). The rate of detection for *C. lukuohia* DNA from the airborne particle samplers ranged from 67% from a site within the heavily infested Waiakea Forest Reserve in South Hilo to as low as 13% at one of the PTA sites mentioned above. *C. huliohia* detections were most frequent at the Konawaena site in South Kona (39%), but they were variable and as low as 0% for four of the 26 total collection sites. None of the five Rotorod samples collected on Molokai Island during November 2020 (where neither *Ceratocystis* species has been detected), nor the 17 samples collected on Maui Island during the same period (where *C. huliohia* has been detected only once), was positive for either *Ceratocystis* species using the assays. Thus, no false positives were detected in either replicate reaction of both qPCR assays from these 22 samples.

These data indicate that fungal particles containing DNA of *C. lukuohia* and *C. huliohia* become windblown over long distances (at least tens of kilometers), thereby supporting the first part of the hypothesis of airborne dispersal. Concurrent research on the infectivity of expelled frass from ambrosia beetle tunneling in trees killed by *Ceratocystis* supports the hypothesis that such ambrosia beetle activity is the likely means of liberation of infectious particles from diseased trees (Hughes et al. 2022; Roy et al. 2019, 2020a), as has been suggested with other *Ceratocystis* species (Harrington 2013). Iton (1960) demonstrated that airborne frass particles of *C. cacaofunesta* from *Theobroma cacao* are infectious, and Luchi et al. (2013) were able to detect airborne *C. platani* DNA with qPCR for hundreds of meters downwind from a site where killed London plane trees were being sawn. However, the viability of particles collected on the greased rods in our samplers was not confirmed in attempts to culture fungi directly from the rods through carrot baiting (data not shown). Successful isolation would confirm long-range, airborne dispersal of infectious frass, but cultivating *Ceratocystis* spp. from minute quantities of spores is a formidable challenge against the heavy background of environmental contaminants (e.g., microbiota, dust), particularly in the absence of selective media for these fungi. Desiccation and DNA damage due to UV irradiation likely impact the viability of

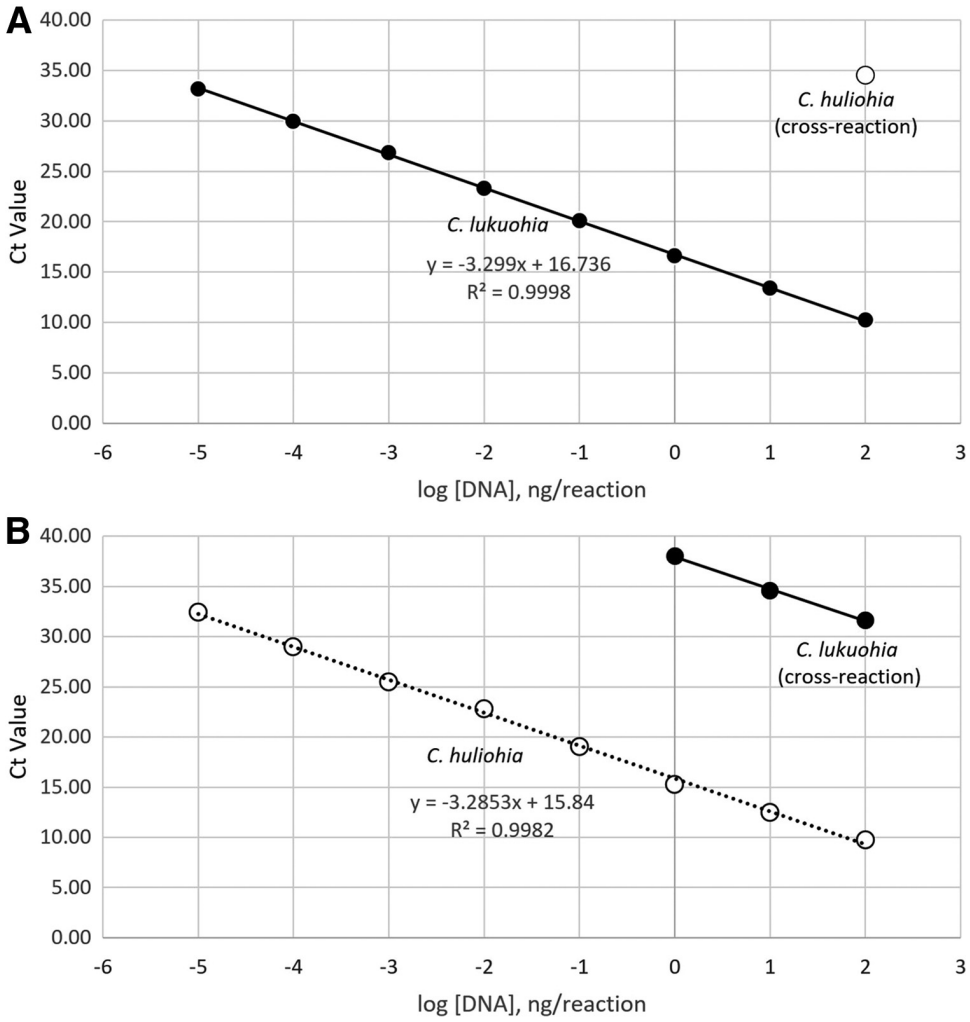


**FIGURE 3**
Standard curves of ITS qPCR assays for *Ceratocystis lukuohia* and *C. huliohia*. Tenfold serial dilutions of purified fungal DNA were used to prepare template from the 100 ng/20 µl reaction to the 1 fg/20 µl reaction. **A,** *C. lukuohia* assay using *C. lukuohia* template (filled circles, solid trendline), with cross-reactivity of *C. huliohia* template (empty circle) shown at the 100 ng/20 µl reaction. **B,** *C. huliohia* assay using *C. huliohia* template (empty circles, dashed trendline), with cross-reactivity of *C. lukuohia* template (filled circles, solid trendline) above the 1 ng/20 µl reaction. Points represent the mean Ct values from three replicate reactions.

airborne spores, and these factors could continue to cause spore mortality even on particles embedded on the collector rods themselves, further complicating the execution of viability studies of particle samples. Survival of *C. lukuohia* in ambrosia beetle frass has been demonstrated (Hughes et al. 2022), but more work is needed to determine if viable spores are capable of dispersing in the wind for long distances.

Any sample for which DNA is amplified, and particularly if the sequence is verified, could be considered a positive detection. However, quantification of trace amounts of DNA is inaccurate. In some of the DNA extracts, a *Ceratocystis* species was detected in one of the replicates at a high Ct value but was not detected in the other replicate. We determined that samples contained the target DNA if at least one of the replicates exceeded the fluorescence threshold within 50 cycles of qPCR, and the presented Ct values are from the mean of the replicates in which there was amplification. The intended application of the method is to make quantitative measurements of DNA, which requires that a minimum threshold is established for usable data. The efficiency of the sampling equipment and efficiency of the DNA extraction method both strongly influence the efficiency of detection. For future experimentation of Rotorod sampling, the number of samplers, their distance from the source, and the sampling interval should be carefully considered. Some insight into these *Ceratocystis* species can be obtained from Atkinson et al. (2019) and Atkinson and Roy (2023).

We tested DNA extracts from 74 soil samples of various compositions for the presence of *C. lukuohia* and *C. huliohia* using the ITS qPCR assays, with many positive detections (Tables 3 and 4). We observed qPCR inhibition of the internal control (higher than expected Ct values for the *M. polymorpha* assay) in only 12% of the total number of tested samples, so the quality and purity of extracted DNA were generally high. For eight positive qPCR detections of each species (those with bold Ct values in Table 3), Sanger sequencing of the cloned PCR products was used to confirm that the sequence of the products matched that of the target species.

Of the 40 individual soil samples collected across Hawaii Island between 11 February 2016 and 8 May 2017, 12 (30%) and 8 (20%) tested positive for *C. lukuohia* and *C. huliohia* DNA, respectively, and no sample resulted in detection of both species (Table 3). Even higher detection rates were found for the two sets of island-wide boot-brush samples collected during summer 2018 (15 May 2018 to 3 September 2018); 57 to 90% were positive for *C. lukuohia* DNA, and 50 to 90% had *C. huliohia* DNA. In addition, a cultured isolate of each species was obtained from these boot-brush soil samples (Table 4): *C. lukuohia* from material collected at Hapuna Beach and *C. huliohia* from material collected from Waipio Valley. Neither of these sites was near a known disease focus. The boot-brush data support the presence of infectious particles for both species in soil and possible intra-island dispersal by foot traffic, and they provide evidence that the sanitation practices advocated in stopping the spread are worthwhile. One possible application for our ITS qPCR assays could be continued and ongoing testing of soil and debris collected from boot brush sanitation stations as they become deployed across the state or more distant locations.

These results confirm the presence of DNA of both *C. lukuohia* and *C. huliohia* in air and soil, and highly sensitive tools were developed for further elucidation of the means of dispersal of these important pathogens. Widespread surveillance of fungal inoculum in both soilborne and windborne forms by ITS qPCR would provide the greatest likelihood of detection of incipient introductions to new geographic locations, and furthermore, the combination of the two datasets collected over long periods would provide valuable information to confirm the hypothesis that air-

**TABLE 2**

Detections of *Ceratocystis lukuohia* and *C. huliohia* DNA in Rotorod samples using the ITS qPCR assays

| | | | *C. lukuohia* | | *C. huliohia* | |
|---|---|---|---|---|---|---|
| Site no.[a] | Site description | Total samples | No. pos. | % pos. | No. pos. | % pos. |
| 1 | Kilauea Military Reserve | 31 | 11 | 35% | 3 | 10% |
| 2 | Waiakea CTAHR Station | 30 | 12 | 40% | 3 | 10% |
| 3 | Waiakea Forest Reserve | 3 | 2 | 67% | 0 | 0% |
| 4 | Leilani Estates | 19 | 3 | 16% | 2 | 11% |
| 5 | Wao Kele o Puna FR | 16 | 4 | 25% | 0 | 0% |
| 6 | Volcano CTAHR Station | 31 | 9 | 29% | 2 | 6% |
| 7 | Volcano Village | 21 | 4 | 19% | 2 | 10% |
| 8 | Olaa Forest Reserve | 19 | 4 | 21% | 0 | 0% |
| 9 | Royal Hawaiian Estates | 15 | 4 | 27% | 1 | 7% |
| 10 | Fern Forest Estates | 17 | 3 | 18% | 1 | 6% |
| 11 | HVNP - Bird Park | 29 | 12 | 41% | 2 | 7% |
| 12 | HVNP - Mauna Ulu | 7 | 1 | 14% | 0 | 0% |
| 13 | HVNP - Hilina Pali | 4 | 1 | 25% | 0 | 0% |
| 14 | HVNP - Footprints | 5 | 2 | 40% | 0 | 0% |
| 15 | Kau - Ranchland | 30 | 8 | 27% | 3 | 10% |
| 16 | Kohala - Ranchland | 40 | 20 | 50% | 13 | 33% |
| 17 | Kohala - Koaia Sanctuary | 44 | 25 | 57% | 13 | 30% |
| 18 | Kohala - DWS | 38 | 22 | 58% | 10 | 26% |
| 19 | Kohala - KWP | 28 | 8 | 29% | 4 | 14% |
| 20 | Mealani CTAHR Station | 36 | 21 | 58% | 8 | 22% |
| 21 | Kalopa State Park | 22 | 7 | 32% | 2 | 9% |
| 22 | Pohakuloa Training Area - TA22 | 16 | 6 | 38% | 4 | 25% |
| 23 | Pohakuloa Training Area - KKE | 8 | 1 | 13% | 1 | 13% |
| 24 | Kona - Kopiko | 29 | 16 | 55% | 7 | 24% |
| 25 | Kona - Konawaena | 33 | 20 | 61% | 13 | 39% |
| 26 | Kona - Honomalino | 9 | 2 | 22% | 3 | 33% |
| | Total | 580 | 228 | 39% | 97 | 17% |

[a] Figure 2 contains a map of sampler locations.

borne particles are the predominant mode of long-distance inoculum dispersal.

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**TABLE 3**

Detections of *Ceratocystis lukuohia* and *C. huliohia* in individual soil samples and boot-brush samples using the ITS qPCR assays

| Sample location | Collection date | ITS qPCR Ct values[a] | | |
|---|---|---|---|---|
| | | *C. lukuohia* | *C. huliohia* | MeNu47[b] |
| Volcano | 21 Dec 2016 | 32 | – | 32 |
| Volcano | 21 Dec 2016 | 32 | – | 32 |
| Volcano | 21 Dec 2016 | 33 | **35**[c] | 32 |
| Volcano | 21 Dec 2016 | **33** | – | 31 |
| Kohala | 6 Nov 2017 | **33** | – | 32 |
| HVNP Boot Brush - Pua'ulu | 8 May 2017 | **35** | – | 31 |
| Panaewa | 7 Dec 2016 | **36** | – | 32 |
| Kohala | 6 Nov 2017 | 36 | – | 32 |
| HVNP - Bird Park | 3 Mar 2016 | **37** | – | 32 |
| Kapoho | 7 Dec 2016 | **37** | – | 32 |
| Waikea Uka | 7 Dec 2016 | **37** | **34** | 32 |
| Honaunau | 29 Mar 2018 | **37** | **36** | 32 |
| Hawaiian Beaches | 7 Dec 2016 | – | **30** | 31 |
| Volcano | 7 Dec 2016 | – | **33** | 31 |
| Honaunau | 29 Mar 2018 | – | **34** | 32 |
| Laupahoehoe | 7 Dec 2016 | – | **35** | 32 |
| Kulani Rd | 7 Dec 2016 | – | **39** | 31 |
| Volcano Village | 11 Feb 2016 | – | – | 32 |
| Volcano Village | 11 Feb 2016 | – | – | 29 |
| Volcano Village | 11 Feb 2016 | – | – | 31 |
| Volcano Village | 11 Feb 2016 | – | – | 31 |
| HVNP - Bird Park | 3 Mar 2016 | – | – | 32 |
| Hilo Watershed Forest Reserve | 1 Dec 2016 | – | – | 31 |
| Hilo Watershed Forest Reserve | 1 Dec 2016 | – | – | 32 |
| Kulani | 7 Dec 2016 | – | – | 32 |
| Keeau | 7 Dec 2016 | – | – | 31 |
| Kukuhaile | 7 Dec 2016 | – | – | 32 |
| Volcano | 7 Dec 2016 | – | – | 32 |
| Puna | 7 Dec 2016 | – | – | 32 |
| Na Ala Hele Boot Brush - Pololu | 21 Dec 2016 | – | – | 31 |
| Na Ala Hele Boot Brush - Puu Huluhulu | 21 Dec 2016 | – | – | 31 |
| Na Ala Hele Boot Brush - Humu Ula | 21 Dec 2016 | – | – | 32 |
| HVNP Boot Brush - Thurston Lava Tube | 8 May 2017 | – | – | 32 |
| HVNP - Volcano House | 8 May 2017 | – | – | 32 |
| Ola'a Forest Reserve | 13 Nov 2017 | – | – | 31 |
| Na Ala Hele Boot Brush - Puna | 21 Dec 2016 | – | – | 35*[d] |
| Kulani Rd | 7 Dec 2016 | – | – | 38* |
| Waimea | 7 Dec 2016 | – | – | –* |
| Puna | 7 Dec 2016 | – | – | –* |
| HVNP Boot Brush - Kilauea Iki | 8 May 2017 | – | – | –* |

[a] Ct values are the estimated number of PCR cycles to detect the target DNA of the respective assay, with higher Ct values correlated with lower amounts of target DNA; – indicates no DNA of the target species detected.
[b] Reactions included a 10 pg spike-in of ohia DNA for internal amplification control to check for inhibition.
[c] Boldface Ct values indicate that the sequence of ITS qPCR amplicon determined by cloning and sequencing matched the target species of *Ceratocystis*.
[d] Asterisk indicates that reactions were partially or completely inhibited.

**TABLE 4**

Summary of island-wide boot brush station detection of *Ceratocystis lukuohia* and *C. huliohia* using ITS qPCR assays and carrot baiting

| Description and collection dates | Total no. | ITS qPCR detections | | | Carrot-baiting isolations | |
|---|---|---|---|---|---|---|
| | | *C. lukuohia* | *C. huliohia* | Inhibited[a] | *C. lukuohia* | *C. huliohia* |
| HVNP Boot Brush Stations (15 May 2018) | 14 | 8 (57%) | 7 (50%) | 2 (14%) | 0 | 0 |
| Na Ala Hele Boot Brush Stations (15 Jun 2018–3 Sep 2018) | 20 | 18 (90%) | 18 (90%) | 2 (10%) | 1 - Hapuna | 1 - Waipio |

[a] Reactions included a 10 pg spike-in of ohia DNA for internal amplification control to check for inhibition. Amplification was partially or completely inhibited in four of the 34 samples.

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## LITERATURE CITED


Atkinson, C. T., and Roy, K. 2023. Environmental monitoring for invasive fungal pathogens of ʻŌhiʻa (*Metrosideros polymorpha*) on the Island of Hawaiʻi. Biol. Invasions 25:399-410.

Atkinson, C. T., Roy, K., and Granthon, C. 2019. Economical environmental sampler designs for detecting airborne spread of fungi responsible for Rapid Ōhia Death. Hawaii Cooperative Studies Unit Technical Report HCSU-087, University of Hawaiʻi at Hilo, Hilo, HI.

Atkinson, C. T., Watcher-Weatherwax, W., Roy, K., Heller, W. P., and Keith, L. M. 2017. A rapid diagnostics test and mobile "Lab-in-a-suitcase" platform for detecting *Ceratocystis* spp. responsible for rapid Ōhia death. Hawaii Cooperative Studies Unit Technical Report HCSU-082, University of Hawaiʻi at Hilo, Hilo, HI.

Barnes, I., Fourie, A., Wingfield, M. J., Harrington, T. C., McNew, D. L., Sugiyama, L. S., Heller, W. P., and Keith, L. M. 2018. Two new species, *Ceratocystis lukuohia* sp. nov. and *Ceratocystis huliohia* sp. nov., associated with the devastating Rapid `Ōhi`a (*Metrosideros polymorpha*) Death (ROD) in Hawai`i. Persoonia 40:154-181.

Brill, E., Hughes, M. A., Heller, W. P., and Keith, L. M. 2019. First report of *Ceratocystis lukuohia* on *Metrosideros polymorpha* on the Island of Kauaʻi, Hawaiʻi. Plant Dis. 103:2961.

Dolezel, J., Bartos, J., Voglmayr, H., and Greilhuber, J. 2003. Nuclear DNA content and genome size of trout and human. Cytometry A 51:127-128.

Fortini, L. B., Kaiser, L. R., Keith, L. M., Price, J., Hughes, R. F., Jacobi, J. D., and Friday, J. 2019. The evolving threat of Rapid ʻŌhiʻa Death (ROD) to Hawaiʻi's native ecosystems and rare plant species. Forest Ecol. Manag. 448:376-385.

Harrington, T. C. 2013. Ceratocystis diseases. Pages 230-255 in: Infectious Forest Diseases. P. Gonthier and G. Nicolotti, eds. CAB International, Wallingford, U.K.

Hawaii Administrative Rules. 2016. Quarantine restrictions on ohia and soil from rapid ohia death infested areas. Amendment §4-72-13.

Hawaii Administrative Rules. 2020. Prohibited Introduction. Amendment §4-70-56.

Heller, W. P., Hughes, M. A., Luiz, B. C., Brill, E., Friday, J. B., Williams, A. M., and Keith, L. M. 2019. First report of *Ceratocystis huliohia* causing mortality of *Metrosideros polymorpha* trees on the Island of Kauaʻi, Hawaiʻi USA. Forest Pathol. 49:e12546.

Heller, W. P., and Keith, L. M. 2018. Real-time PCR assays to detect and distinguish the rapid ʻōhiʻa death pathogens *Ceratocystis lukuohia* and *C. huliohia*. Phytopathology 108:1395-1401.

Hughes, M. A., Juzwik, J., Harrington, T. C., and Keith, L. M. 2020. Pathogenicity, symptom development, and colonization of *Metrosideros polymorpha* by *Ceratocystis lukuohia*. Plant Dis. 104:2233-2241.

Hughes, M. A., Roy, K., Harrington, T. C., Brill, E., and Keith, L. M. 2022. *Ceratocystis lukuohia*-infested ambrosia beetle frass as inoculum for Ceratocystis wilt of ohia (*Metrosideros polymorpha*). Plant Pathol. 72:232-245.

Iton, E. F. 1960. Studies on a wilt disease of cacao at River Estate. II. Some aspects of wind transmission. Pages 47-58 in: Annual Report on Cacao Research, 1959-60. St. Augustine, Trinidad: Imperial College of Tropical Agriculture, University of West Indies.

Keith, L. M., Hughes, R. F., Sugiyama, L. S., Heller, W. P., Bushe, B. C., and Friday, J. B. 2015. First report of *Ceratocystis* wilt on `Ōhi`a (*Metrosideros polymorpha*). Plant Dis. 99:1276.

Luchi, N., Ghelardini, L., Belbahri, L., Quartier, M., and Santini, A. 2013. Rapid detection of *Ceratocystis platani* inoculum by quantitative real-time PCR assay. Appl. Environ. Microbiol. 79:5394-5404.

Luiz, B., and Keith, L. M. 2021. Influence of culture media and temperature on growth and sporulation of *Ceratocystis lukuohia*. Pacific Sci. 74:389-394.

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

Moller, W. J., and Devay, J. E. 1968. Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. Phytopathology 58:123-124.

Mortenson, L. A., Hughes, R. F., Friday, J. B., Keith, L. M., Barbosa, J. M., Friday, N. J., Liu, Z., and Sowards, T. G. 2016. Assessing spatial distribution, stand impacts and rate of *Ceratocystis fimbriata* induced ʻōhiʻa (*Metrosideros polymorpha*) mortality in a tropical wet forest, Hawaiʻi Island, USA. Forest Ecol. Manag. 377:83-92.

Perroy, R. L., Sullivan, T., Benitez, D., Hughes, R. F., Keith, L. M., Brill, E., Kissinger, K., and Duda, D. 2021. Spatial patterns of ʻŌhiʻa mortality associated with Rapid ʻŌhiʻa Death and ungulate presence. Forests 12:1035.

Pilotti, M., Lumia, V., Di Lernia, G., and Brunetti, A. 2012. Development of real-time PCR for in wood-detection of *Ceratocystis platani*, the agent of canker stain of *Platanus* spp. Eur. J. Plant Pathol. 134:61-79.

Roy, K., Ewing, C. P., Hughes, M. A., Keith, L., and Bennett, G. M. 2019. Presence and viability of *Ceratocystis lukuohia* in ambrosia beetle frass from Rapid ʻŌhiʻa Death-affected *Metrosideros polymorpha* trees on Hawaiʻi Island. Forest Pathol. 49:e12476.

Roy, K., Jaenecke, K. A., Bjontegard, N., Mikros, D., Dunkle, E. J., Yanger, C., Sugiyama, L. S., Keith, L. M., and Peck, R. W. 2020b. Decontamination of *Ceratocystis* pathogens responsible for Rapid ʻŌhiʻa Death. Plant Health Prog. 21:301-305.

Roy, K., Jaenecke, K. A., and Peck, R. W. 2020a. Ambrosia beetle (*Coleoptera*: *Curculionidae*) communities and frass production in ʻŌhiʻa (*Myrtales*: *Myrtaceae*) infected with *Ceratocystis* (*Microascales*: *Ceratocystidaceae*) fungi responsible for Rapid ʻŌhiʻa Death. Environ. Entomol. 49:1345-1354.

Vaughn, N. R., Asner, G. P., Brodrick, P. G., Martin, R. E., Heckler, J. W., Knapp, D. E., and Hughes, R. F. 2018. An approach for high-resolution mapping of Hawaiian *Metrosideros* forest mortality using laser-guided imaging spectroscopy. Remote Sensing 10:502.