

First report of *Ceratocystis huliohia* causing mortality of *Metrosideros polymorpha* trees on the Island of Kaua'i, Hawai'i USA

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Abstract

The fungus *Ceratocystis huliohia* was detected for the first time in dead and dying *Metrosideros polymorpha* trees on the Island of Kaua'i, Hawai'i, U.S.A. Fungal isolates were recovered from diseased trees into culture, and identities were confirmed by diagnostic qPCR assay and DNA sequencing. Growth chamber inoculations confirmed *C. huliohia* pathogenicity on *M. polymorpha* seedlings and subsequently the same fungus was recovered, completing Koch's postulates. DNA sequence analysis revealed a unique ITS haplotype for Kaua'i isolates of *C. huliohia* that has not been found on Hawai'i Island.

KEYWORDS

detection, ITS haplotype, rapid ohia death

1 | INTRODUCTION

Rapid 'ōhi'a death (ROD) is responsible for widespread mortality to *Metrosideros polymorpha* Gaudich ('ōhi'a lehua) tree populations on Hawai'i Island and is caused by two newly described fungal pathogens, *Ceratocystis lukuohia* and *Ceratocystis huliohia* Barnes et al. (2018). Due to concerns over potential inter-island spread or undetected cryptic infestations, land managers are monitoring for ROD-induced mortality on six main islands across the state. Herein, we report *C. huliohia* causing *M. polymorpha* mortality on the island of Kaua'i in the State of Hawai'i using molecular, culture and pathogenicity assays. This is the first detection of the fungus outside of Hawai'i Island. Phylogenetic analysis shows that fungal isolates from Kaua'i are distinct from those of Hawai'i Island at the ITS locus.

2 | MATERIALS AND METHODS

2.1 | Initial sampling and diagnostics

During two field visits in April–May 2018, several (15+) *M. polymorpha* trees in the Moloa'a Forest Reserve of northeast Kaua'i were

observed with symptoms ranging from partial branch dieback to completely dead and defoliated canopies (Figure 1a). Using hatchets or cordless drills with bits, the sapwood of diseased trees was collected, sealed in plastic bags and sent to the USDA Agricultural Research Service (ARS), Daniel K. Inouye U.S. Pacific Basin Agricultural Research Center (PBARC) in Hilo (HI) for diagnostic testing. Sixteen trees were sampled, three of which were sampled twice for secondary confirmation. DNA was extracted from the wood shavings and screened for the causal agents of ROD, *C. lukuohia* and *C. huliohia*, using a diagnostic multiplex qPCR assay (Heller & Keith, 2018). Isolation of the pathogen from outer sapwood tissue was attempted by carrot baiting (Moller & DeVay, 1968). Primary fungal isolations were made by transferring a single perithegium formed on carrot discs onto 10% V8 agar plates amended with 10% streptomycin and incubated at 25°C.

2.2 | DNA sequencing

DNA from wood samples and fungal isolates was prepared using a commercial spin-column extraction kit (Macherey-Nagel

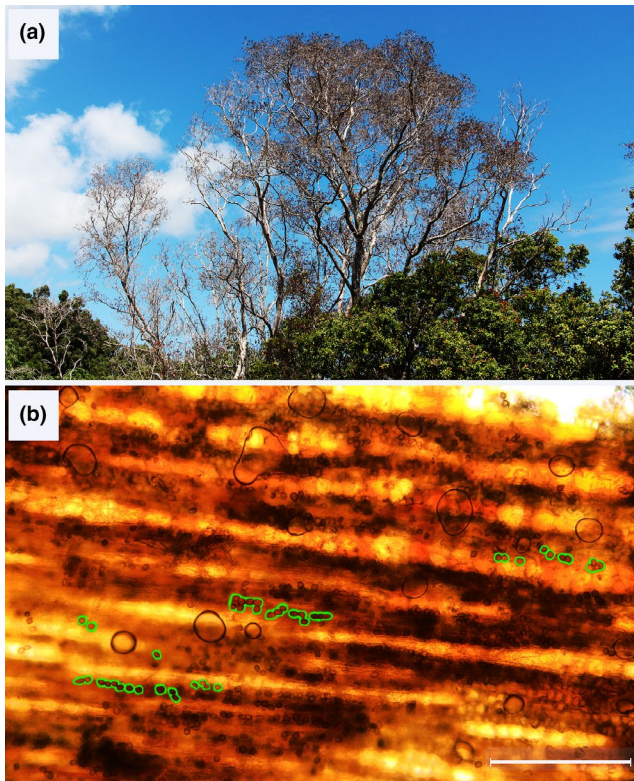


FIGURE 1 Symptoms and signs of *Ceratocystis huliohia* infection of *Metrosideros polymorpha* trees. (a) Wilted and partially defoliated *M. polymorpha* tree in the Moloa'a Forest Reserve of northeast Kaua'i. (b) Thin wood sections of *C. huliohia*-inoculated seedling with abundant aleurioconidia (green highlighting) within xylem vessels. Image was captured under 100 \times magnification and scale bar represents 250 μ m

NucleoSpin Plant II Kit). Verification of the qPCR detections of *C. huliohia* from wood DNA extracts was performed using primers CeraF and CeraR (Luchi, Ghelardini, Belbahri, Quartier, & Santini, 2013), a genus-specific primer combination targeting the cerato-platanin gene that has been found to work well on complex DNA mixtures of plant and fungal DNA, such as decaying wood tissue. Once pure isolates were acquired, the cerato-platanin fragment, plus five additional loci were amplified from two isolates, P18-81 and P18-82. The additional amplicons included ribosomal DNA ITS (White, Bruns, Lee, & Taylor, 1990), beta-tubulin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin and translation elongation factor-1 alpha (*TEF-1 α*) (Heller & Keith, 2018). Additionally, because the ITS sequence of the Kauai isolates differed from that of the published Hawai'i Island isolates, ITS was amplified and sequenced for the remaining 43 Hawai'i Island *C. huliohia* isolates in the USDA-PBARC collection.

GoTaq DNA polymerase (Promega, Madison, WI) was used for all amplifications using cycling parameters: initial denaturation of 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a final extension of 5 min at 72°C. PCR products were cleaned up using ExoSAP-IT (ThermoFisher) and submitted for bulk Sanger DNA sequencing (Eurofins Genomics).

2.3 | Koch's postulates

Pathogenicity tests were conducted on 2-year-old *M. polymorpha* seedlings (approx. 0.5 m tall) in growth chambers (Conviron) set to a 12 hr day/night cycle at 24°C. Inoculum was made with a single *C. huliohia* isolate (P18-81) recovered from a diseased 'ōhi'a in Kaua'i with inoculation methods similar to Barnes et al. (2018). In brief, sterile filter paper discs (5 mm diameter) were soaked in a *C. huliohia* conidial suspension (1.1 and 1.3×10^7 conidia/ml for experiments 1 and 2, respectively) for 1 min, plated on 10% V8 agar and incubated for 4 days at 24°C until thoroughly colonized. 'Ōhi'a plants were inoculated by creating a vertical wound into the stem to reach the xylem tissue roughly 2 cm above the soil using a sterile scalpel. The inoculated filter paper disc was inserted into the wound, the stem-flap was closed and wrapped with a wet cotton ball held in place by wire. The cotton ball was moistened daily to prevent desiccation of the inoculation site. *Ceratocystis huliohia* was inoculated into four plants, and two control plants were inoculated with filter paper discs soaked in sterile distilled water. Plants were maintained in a growth chamber and checked for symptoms and irrigated three times a week. At seedling death or 70 days post-inoculation, each plant was de-barked and visually assessed for the presence of vascular discoloration and pathogen recovery was attempted by carrot baiting. This experiment was conducted twice for a total of eight inoculated seedlings and four controls ($n = 12$ total).

3 | RESULTS

Ceratocystis huliohia was detected by qPCR in the wood of 11 of 16 trees sampled from the Moloa'a Forest Reserve. Trees in which *C. huliohia* DNA was detected in the first sampling trip (April 2018) were re-sampled and confirmed as positive in May 2018. Carrot baits yielded viable *C. huliohia* from five of 16 samples. The DNA sequences of two Kaua'i *C. huliohia* isolates (P18-81, P18-82) for cerato-platanin, beta-tubulin, actin, translation elongation factor-1 alpha and glyceraldehyde-3-phosphate dehydrogenase were 100% identical to those reported for Hawai'i Island *C. huliohia* isolates: GenBank accessions KU043258, KU043264, KU043246, KU043290 and KU043273, respectively (Heller & Keith, 2018). The ribosomal DNA ITS sequence for all five Kaua'i *C. huliohia* isolates recovered was identical and deposited as GenBank accession MH996461. The Kaua'i isolate ITS sequence differs from the published sequence of ten Hawai'i Island isolates (Barnes et al., 2018; Heller & Keith, 2018). Within the 615 bp primer-trimmed ITS5/ITS4 PCR amplicon alignment, two indels distinguish the Kaua'i and Hawai'i Island ITS genotypes: an apparent TT insertion at position 166–167 and a T deletion at position 396 within the Hawai'i isolate sequence relative to the Kaua'i isolate sequence (Figure 2). To confirm the Kaua'i isolate sequence was not also present on Hawai'i Island, the remainder of the USDA-PBARC collection (= 43 additional *C. huliohia* isolates) was screened by PCR and sequencing; all additional isolates contained the Hawai'i Island ITS genotype (100%

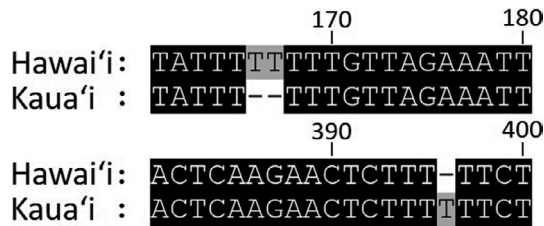


FIGURE 2 Partial ITS alignment showing the polymorphisms present between Hawai'i Island and Kaua'i *Ceratocystis huliohia* isolates. Numbers indicate relative positions of primer-trimmed ITS5/4 PCR products; dashes indicate gaps

match to GenBank KU043252). The complete list of isolate numbers and their source locations are shown in Appendix S1.

During the first pathogenicity trial, two of four *C. huliohia*-inoculated seedlings developed wilt and died within 70 days post-inoculation. Upon dissection, extensive vascular staining was visible in the xylem of all fungus-inoculated plants, including the two plants which were externally asymptomatic. Abundant aleuroconidia were visible in thin xylem strips under 100X magnification (Figure 1b). In the second trial, all inoculated plants wilted and died within 70 days and had visible vascular staining along the main stem. All control plants remained healthy and had no observable vascular staining. A fungus of *Ceratocystis huliohia*-type morphology was recovered by carrot baiting for all inoculated plants, but not for controls. A single, fungus-colonized carrot bait per experiment was analysed by qPCR and *C. huliohia* was identified molecularly, thus confirming the fungal identity as *C. huliohia* and completing Koch's postulates.

4 | DISCUSSION

ITS sequences aside, *C. huliohia* from Hawai'i Island and Kaua'i have both been shown to be pathogens of 'ōhi'a in our laboratory tests and have both been isolated from naturally infected, mature, dead 'ōhi'a trees. While it is not uncommon for individual *Ceratocystis* species to have multiple ITS haplotypes (Harrington, Kazmi, Al-Sadi, & Ismail, 2014), the mutually exclusive distribution of the two *C. huliohia* ITS genotypes on Kaua'i and Hawai'i Island suggests that the introduction of the pathogen to each island was probably not directly from the other island. Ongoing work focuses on tracking the movement of ROD populations across landscapes using available genetic markers; however, the common link between the Kaua'i and Hawai'i Island *C. huliohia* populations is yet to be found. Furthermore, the exact origin of the *C. huliohia* species and its mode of transmission to Hawai'i also remains elusive. For now, the best management practices to prevent the spread of ROD include rapid detection, sanitation, preventing wounds on 'ōhi'a and limiting the movement of infected wood within and between islands.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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