

SHORT COMMUNICATION

Presence and viability of *Ceratocystis lukuohia* in ambrosia beetle frass from Rapid 'Ōhi'a Death-affected *Metrosideros polymorpha* trees on Hawai'i Island

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Abstract

Rapid 'Ōhi'a Death (ROD) is a fungal disease of 'ōhi'a lehua (Myrtaceae: *Metrosideros polymorpha*) caused by *Ceratocystis lukuohia* and *C. huliohia*. ROD is the aetiological agent of widespread mortality of this important tree on Hawai'i Island, but its epidemiology remains unclear. We investigated the prevalence and viability of *C. lukuohia* in ambrosia beetle frass in ROD-affected 'ōhi'a trees. A total of 200 frass traps were placed onto *C. lukuohia*-infected 'ōhi'a at four locations on the east side of Hawai'i Island. Frass was collected and screened for the presence of *C. lukuohia* DNA using a diagnostic qPCR assay. In addition, frass samples were screened for viability by carrot baiting. All trapped beetles were of the genus *Xyleborus*, with the majority being the non-native *X. ferrugineus*. Of the frass samples tested, 62% contained *C. lukuohia* DNA and 17% of carrot baits were positive for the fungus. These results indicate that ambrosia beetle frass releases *C. lukuohia* into the environment. We discuss the potential role infested frass could play in the ROD pathosystem.

1 | INTRODUCTION

Rapid 'Ōhi'a Death (ROD) is a recently emerged phenomenon that is decimating the 'ōhi'a lehua (*Metrosideros polymorpha* Gaudich) trees on Hawai'i Island, HI, USA. 'Ōhi'a is the dominant native tree species of the Hawaiian archipelago and is culturally and ecologically important. Since its initial emergence in 2010 in the District of Puna (southeast Hawai'i), the disease has now spread island-wide. However, ROD dispersal between trees and over longer distances

is poorly understood. To develop effective management strategies, better understanding of the disease's epidemiology is needed.

Two novel and exotic fungal species, *Ceratocystis lukuohia* and *C. huliohia* I. Barnes, T.C. Harrin. and L.M. Keith cause ROD, which is characterized by crown wilt and mortality of 'ōhi'a trees (Barnes et al., 2018). *Ceratocystis lukuohia* is the more aggressive of the two species and is associated with several hotspots of expanding tree mortality on the island (Figure 1a) (Barnes et al., 2018; Heller & Keith, 2018); thus, it is the target of this study. Phylogenetically, *C. lukuohia* is a member of the Latin American Clade (LAC) of *Ceratocystis* (*sensu lato*), which includes several aggressive pathogens of hardwoods like

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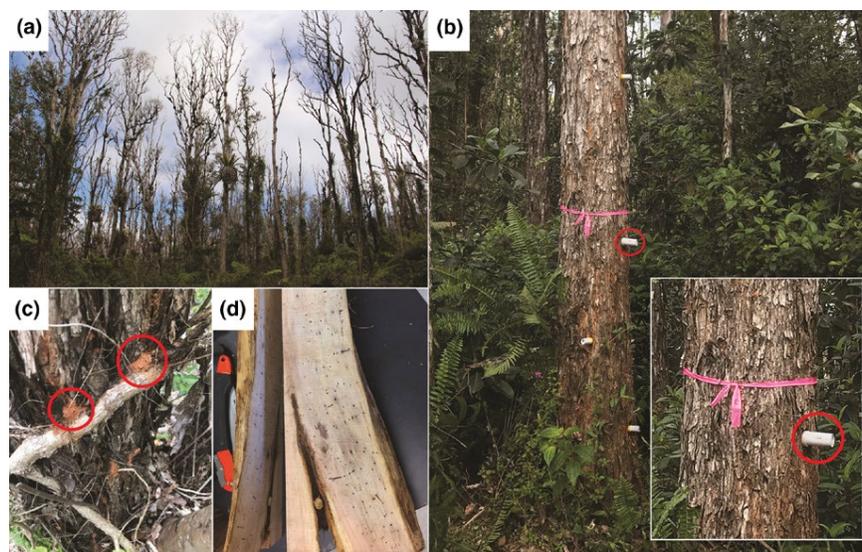


FIGURE 1 Symptoms of Rapid 'Ōhi'a Death in Hawai'i (a) standing dead 'Ōhi'a stems after mortality in the Puna District, HI, (b) frass trap placement on tree bole, (c) ambrosia beetle frass accumulated in vines and bark cracks, and (d) split log showing numerous ambrosia beetle boring holes. Photos: A, D by J.B. Friday University of Hawai'i Mānoa

C. platani (J.M. Walter) Engelbr. and T.C. Harr. (canker stain of plane tree) and *C. cacaofunesta* Engelbr. and T.C. Harr. (mal de machete on cacao) (Barnes et al., 2018; Harrington, 2013). In general, *Ceratocystis* enters woody hosts through wounds, and inoculum can be disseminated by contaminated insects, tools, soil, sawdust and ambrosia beetle frass (Harrington, 2013). On Hawai'i Island, we hypothesize that trunk and branch injury caused by animals, humans, machinery and strong winds creates infection courts for ROD pathogens.

Similar to mango wilt disease in Brazil and *Ceratocystis* wilt of cacao in Trinidad, contaminated ambrosia beetles (Curculionidae: Scolytinae) and their frass are suspected means of dispersal for ROD in Hawai'i (Figure 1c) (Iton, 1961; Souza et al., 2013). Beetles create extensive natal galleries throughout the sapwood of 'Ōhi'a trees, and through their burrowing activities, can come into direct contact with the pathogens. However, the role of these beetles in the persistence and spread of ROD remains unknown. Here, we test the fundamental question of whether the boring activities of ambrosia beetles in ROD-affected 'Ōhi'a release viable *C. lukuohia* propagules (i.e., conidia and/or hyphal fragments) in frass.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Study sites were chosen that had verified ROD mortality in the immediate vicinity. Individual diseased trees were selected based on the presence of symptomatic crowns displaying wilt, die-back, defoliation and active ambrosia beetle infestation. To confirm *Ceratocystis*, ambrosia beetle frass was obtained from each tree and screened for the presence of pathogen DNA using a TaqMan qPCR assay (Applied Biosystems; Heller & Keith, 2018).

Five trees that tested positive for *C. lukuohia* and not *C. huliiohia* were chosen at each of the four sites, for a total of 20 'Ōhi'a trees. Diameter at Breast Height (DBH) was measured for each tree. Four study sites on the eastern side of Hawai'i Island (District of Puna) were selected: Hawaiian Acres (19.5239°N, -155.0395°W), Blacksands Estates (19.4162°N, -154.9584°W), Orchidlands Estates 1 (19.5422°N, -155.0122°W) and Orchidlands Estates 2 (19.5617°N, -155.0178°W). All sites had similar microclimates with 180–300 m elevation, 3,000–4,000 mm rain/year and mean temperatures of $28 \pm 3.2^\circ\text{C}$.

Frass traps were attached to trees between February 17th and March 2nd, 2017. Traps were constructed from 25 ml screw cap tubes (Sarstedt, Germany) painted white with a mesh window to allow airflow at the bottom of the traps (Figure 1b). Bark and outer wood were removed in the area around beetle galleries using a mallet and chisel to flatten the tree surface, providing a seal against water infiltration. Caps with a centred 6 mm hole were affixed directly over the gallery entrance with screws. To avoid cross-contamination, a single trap was used for each beetle gallery. A total of 200 traps were set: 10 traps per tree from 0–3.6 m along each tree bole (Figure 1b). Galleries were chosen based on observations of freshly produced ambrosia beetle frass. Fourteen days after placement, frass and ambrosia beetles were collected from traps and immediately processed. Traps were left over galleries until, at minimum, a single beetle emerged per trap, permitting identification of the species involved in frass production. In some cases, insufficient material was obtained so an additional collection was made after 14 days to obtain a sufficient sample for molecular and viability testing. Beetle specimens were determined to species according to Samuelson (1981) and further verified against online databases and research collections at the Bernice P. Bishop Museum and University of Hawai'i Insect Museum, Honolulu, HI.

2.2 | DNA and viability testing

Ten to 15 mg of frass from each trap was used for DNA extractions. Extractions were carried out with a NucleoSpin Plant II extraction kit (Macherey–Nagel). A TaqMan-based qPCR assay (Applied Biosystems) was used to screen for *C. lukuohia* DNA, following Heller and Keith (2018). The remainder of the frass collected was used for viability testing.

To assess pathogen viability, ambrosia beetle frass was baited on carrot discs and incubated at room temperature for 2–8 weeks (Moller & DeVay, 1968). Carrot discs were scored as *Ceratocystis* positive when greyish fungal mats with perithecia fruiting bodies were observed. After 4 weeks, negative samples were re-hydrated with sterile water to encourage fungal development of recalcitrant samples. To confirm that carrot discs were colonized by *C. lukuohia*, mycelia or ascospore droplets from perithecial necks were collected with a sterile pick and placed onto 10% V8 agar plates amended with streptomycin. Fungal DNA was extracted from an agar plug from all plates and verified via qPCR.

2.3 | Data analysis

Statistical analysis was conducted in R version 3.5.1. Tree DBH means were compared across sites using a one-way ANOVA followed by a Tukey's HSD post hoc test. A mixed effects logistic regression model accounting for the random effect of individual trees and DBH within sites was used to assess whether site location affected the number of positive DNA detections and viability of *C. lukuohia* in frass samples. For the molecular tests, the number of positive qPCR detections was the dependent variable, and site location was the independent variable. Similarly, for viability assays, positive carrot cultures and site location were the dependent and independent variables, respectively. Sample means with \pm standard error estimates are reported below.

3 | RESULTS

Of the 200 frass traps set in this study, 160 produced enough material for qPCR testing and 144 were assayed with carrot baits. *Xyleborus ferrugineus* Fabricius emerged from 157 traps, and *X. perforans* Wollaston emerged from three of the traps. Mean DBH of

trees across all sites was 28.04 ± 2.12 cm. Orchidlands Estates 1 had the largest 'ōhi'a trees ($p < 0.001$) (Table 1). Sixty-two percent of the frass samples tested positive for *C. lukuohia* (Table 1). Positive samples were collected from a range of 10.2–318.8 cm in height along the tree bole. The mean bore-hole height of positive DNA detections was 149.56 ± 9.04 cm from the ground. The number of qPCR detections was not significantly different among fields sites ($p = 0.06$) after accounting for the effect of trees within sites and DBH.

Seventeen percent of all frass samples contained viable *C. lukuohia* propagules (Table 1). Similar to the qPCR results, viable *Ceratocystis* propagules were collected from heights between 19.81–292.10 cm, with the majority of the detections located at 151.02 ± 15.59 cm. The number of viable frass samples differed significantly among sites after accounting for the effect of trees within sites and DBH ($p < 0.001$).

4 | DISCUSSION

The majority of the ambrosia beetles emerging from 'ōhi'a trees in our sites were the adventive species, *X. ferrugineus*. These data suggest *X. ferrugineus* is the dominant species in the Puna area during Spring. Beetle community diversity may experience seasonal shifts, and further research is necessary to better understand the ambrosia beetle species involved in this pathosystem. *Ceratocystis lukuohia* was identified more often by qPCR than via carrot baiting, suggesting that propagule viability may vary outside ROD-affected trees. Although the artificial environment of the emergence traps may contribute to fungal mortality, 17% of our samples were viable. This result indicates that frass may have the potential to start new infections in additional trees. However, viability on carrot baits does not necessarily equate with the ability to cause disease, and the survivability and infectivity of spores in frass remain unknown. Our results are also likely to be a conservative estimate of prevalence and viability; we placed ten frass traps per tree, but diseased 'ōhi'a trees may contain dozens to hundreds of galleries throughout the entire bole (Figure 1d).

Our results also revealed that particular sites had trees that appear to produce more contaminated and viable frass than others. The Orchidlands Estates 1 site had more qPCR detections than any other site, which may be correlated with the statistically significant larger tree diameter at this site (Table 1). The Orchidlands Estates 2

TABLE 1 Presence and viability of *Ceratocystis lukuohia* by qPCR and carrot baiting assays, respectively

| Study Site | Average DBH (\pm SE cm) | qPCR detections | Viability (by carrot bait) |
|-----------------------|----------------------------|-----------------|----------------------------|
| Hawaiian Acres | 24.6 ± 1.96^{ab} | 23/40 (58%) | 1/29 (3%) |
| Blacksands Estates | 28.77 ± 3.02^b | 23/39 (59%) | 5/39 (13%) |
| Orchidlands Estates 1 | 40.6 ± 1.84^c | 33/41 (80%) | 6/40 (15%) |
| Orchidlands Estates 2 | 18.18 ± 1.44^a | 20/40 (50%) | 13/36 (36%) |
| Total | NA | 99/160 (62%) | 25/144 (17%) |

Note. Significantly different means for DBH determined by Tukey's HSD are denoted by a superscript, means that do not share a letter are significantly different at $p < 0.05$.

site had more carrot-bait detections than any other site, which may be attributed to more recent ROD infections. The temporal scale of *C. lukuohia* infection and spore type in frass may also play an important role in the disease. Further research is needed to understand and disentangle these parameters.

Our study shows that viable *C. lukuohia* propagules are released from infected host trees into the environment in the frass of *Xyleborus* spp. ambrosia beetles. These propagules potentially contribute to air- and soil-borne inoculum, which may be important mechanisms of dispersal of ROD fungi within and between 'ōhi'a stands, and possibly over longer distances (e.g., within and between islands). Although our study provides important initial considerations for ROD epidemiology, further work is needed to demonstrate the infectivity of contaminated frass and the circumstances under which this putative inoculum could move to uninfected 'ōhi'a trees.

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REFERENCES

- Barnes, I., Fourie, A., Wingfield, M. J., Harrington, T. C., McNew, D. L., Sugiyama, L. S., ... Keith, L. M. (2018). New *Ceratocystis* species associated with rapid death of *Metrosideros polymorpha* in Hawai'i. *Persoonia*, 40, 154–181. <https://doi.org/10.3767/persoonia.2018.40.07>
- Harrington, T. C. (2013). *Ceratocystis* Diseases. In P. Gonthier, & G. Nicolotti (Eds.), *Infectious forest diseases* (pp. 230–255). Oxfordshire, England: CABI. <https://doi.org/10.1079/9781780640402.0000>
- Heller, W. P., & Keith, L. M. (2018). Real-time PCR assays to detect and distinguish the rapid 'ōhi'a death pathogens *Ceratocystis lukuohia* and *Ceratocystis huliohia*. *Phytopathology*. <https://doi.org/10.1094/PHYTO-09-17-0311-R>
- Iton, E. F. (1961). Studies on a wilt disease of Cacao at River Estate. II. Some aspects of wind transmission. Report on Cacao Research, 47–58.
- Moller, W. J., & DeVay, J. E. (1968). Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology*, 58, 123–126.
- Samuelson, G. A. (1981). A synopsis of Hawaiian Xyleborini (Coleoptera: Scolytidae). *Pacific Insects*, 23, 50–92.
- Souza, A. G. C., Maffia, L. A., Murta, H. M., Alves, Y. H., Pereira, R. M., & Picanço, M. C. (2013). First report on the association between *Ceratocystis fimbriata*, an agent of Mango Wilt, *Xyleborus affinis*, and the sawdust produced during beetle colonization in Brazil. *Plant Disease*, 97, 1116–1116. <https://doi.org/10.1094/PDIS-12-12-1204-PDN>

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