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The trail less traveled: Envisioning a new approach to identifying key food resources for threatened Hawaiian arboreal snails

Wallace M. Meyer III[1], Kenneth A. Hayes[2,3,4], Norine W. Yeung[3,4,5], Edward J. Crane III[1], Alexandra Turvey[1], Claire LeBlanc[1], Andre R. O. Cavalcanti[1]

1 *Pomona College, Biology Department, 175 W. Sixth St., Claremont, CA 91711, USA* **2** *Bishop Museum, Pacific Center for Molecular Biodiversity, 1525 Bernice St., Honolulu, HI 96817, USA* **3** *Smithsonian Institution, National Museum of Natural History, PO Box 37012, MRC 163, Washington, DC, 20013, USA* **4** *Pacific Biosciences Research Center, University of Hawaii, Honolulu, HI 96822, USA* **5** *Bishop Museum, Malacology, 1525 Bernice St., Honolulu, HI 96817, USA*

Corresponding author: Wallace M. Meyer III (wallace_meyer@pomona.edu)

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Abstract

Our understanding of Hawaiian arboreal snails' diets remains rudimentary, hindering the development of effective conservation strategies. To identify important food resources, we tested the hypothesis that epiphytic microbial assemblages differ on plant species preferred and avoided by snails at Mt. Kaala Natural Area Reserve, where snail plant preferences are known from previous studies. Comparing microbial assemblages on plants that snails both prefer and avoid was identified as a potentially key step to moving research away from characterizing which microbes snails encounter, towards testing if microbial assemblages are driving snail plant preferences. We found that fungal and bacterial assemblages differed between plant species preferred and avoided by snails, indicating that Hawaiian arboreal snails may be selecting plants based on their epiphytic microbial assemblages. Previous microbes thought to be important, *Cladosporium* spp., propagated in captive rearing facilities, and *Botryosphaeria* spp., preferred fungi in a feeding experiment, were both rare and had similar abundances on preferred and avoided plant species in Mt. Kaala. Our approach, conducting preference studies before isolating microbes, is key to identifying arboreal snail food resources and improves our ability to identify microbes that form the foundation of Hawaiian arboreal snails' diet. If we can identify important food resources, it greatly expands our ability to: (1) assess and monitor habitat quality, (2) make informed restoration recommendations, and (3) improve rearing efforts for highly endangered captive reared populations.

Keywords
bacteria, conservation, diversity, epiphytic, fungi, gastropod, microbe, Pacific, trophic

Introduction

The Hawaiian archipelago is a land snail diversity hotspot with high richness (759 described species), endemism (> 99%), and extinction rates (Cowie 1995; Cowie et al. 1995; Yeung and Hayes 2018). Most estimates of extinction suggest that > 65% of the Hawaiian land snail species have already been lost and families have already been extirpated from the islands (Solem 1990; Cowie 1998, 2001; Lydeard et al. 2004; Yeung and Hayes 2018). Since many of the remaining species continue to experience reductions in range and population sizes, effective conservation strategies are urgently needed (Solem 1990; Yeung and Hayes 2018). Unfortunately, developing effective management strategies for Hawaiian snails, and most threatened invertebrates, is often hindered by a lack of key ecological information (Lydeard et al. 2004; Cardoso et al. 2011; Yeung and Hayes 2018).

While many factors contribute to the limited ecological information on threatened invertebrates (see Cardoso et al. 2011; Regnier et al. 2015; Cowie et al. 2022), unraveling the ecology of many invertebrate species can be difficult. For example, our understanding of what Hawaiian arboreal snails, i.e., snails that live primarily on trees and shrubs, eat remains rudimentary despite significant research effort (see references below). Hawaiian arboreal snails graze on the fungi, bacteria, and algae on the surface of plants, hereafter, referred to as the phyllosphere. As the phyllosphere is composed of extremely small (often single-celled) organisms and phyllosphere assemblages are hyper-diverse (i.e. thousands of different fungi and bacteria on a single plant), determining which food resources are important can be extremely difficult. However, identifying important food resources is key to assessing habitat quality, informing restoration efforts aimed at preserving the remaining Hawaiian land snail species, and developing cultures to enhance fitness and limit exposure to pathogens and/or toxins for captively reared endangered snails (Sischo et al. 2016; Strouse et al. 2021; Meyer et al. 2022).

Previous research has used a variety of approaches to identify important arboreal snail food resources, but each has shortfalls. One approach examines differences in survivorship, growth, and reproductive output when snails are offered different food resources (Holland et al. 2017; Strouse et al. 2021). For example, Holland et al. (2017) found that while survivorship was not impacted, egg production by *Auriculella diaphana* was reduced by 15 to 20% when snails grazed on non-native plant surfaces as opposed to native plants. Similarly, Strouse et al. (2021) found that *A. diaphana* produced 2.4 and 30.1 times more eggs when provided both native vegetation and a lab reared fungus relative to treatments where snails were provided just native vegetation or lab reared fungus alone. While these experiments suggest that phyllospheres on native plants can enhance the fecundity of *A. diaphana*, we are left with little information

on which components of the phyllosphere are important, if patterns hold for other native and non-native plants, and if this snail species is a good model for other Hawaiian land snails. For example, the non-native ginger, *Hedychium coronarium*, used by Holland et al. (2017), was preferred by native succineids on the island of Hawaii, and reproduction on ginger in the wild seemed robust (Brown et al. 2003, 2006; Meyer 2012). Similarly, *Metrosideros polymorpha*, a widespread native tree species used by both Holland et al. (2017) and Strouse et al. (2021), was avoided by all snail species in native wet cloud-forests on Maui, Hawaii, and Oahu (Meyer 2012; Meyer et al. 2014, 2022), though *M. polymorpha* was a preferred plant for two endangered tree snails, *Achatinella sowerbyana* and *Achatinella lila* in forests with native and non-native vegetation on Oahu (Price et al. 2017). A second approach uses metagenomic techniques to characterize phyllospheres on plants used by native snails and to compare the microbial assemblages on leaves to those in fecal pellets in an effort to determine what they are eating (O'Rorke et al. 2015; Price et al. 2017). Assessing the phyllosphere of plants that act only as snail hosts has limited utility as comparisons are required to explore why certain plants and phyllospheres may be chosen. Comparing microbial assemblages between fecal and leaf samples also has limitations, as elevated abundances of some microbes in the fecal samples may suggest that microbes are important food resources targeted by the snails, or that snails are unable to assimilate those microbes. Alternatively, snail feces may act as a colonizing substrate that is facilitating the growth of microbial assemblages that have nothing or little to do with their diet. Feeding trials have also been used in a limited capacity. O'Rorke et al. (2016) explored preferences among 11 microbial (bacterial and fungal) isolates obtained from leaf and fecal samples using feeding trials. While isolating targeted microbes is difficult, extrapolating O'Rorke et al.'s (2016) findings that Potato Dextrose Agar (PDA) media and *Cladosporium* spp., a fungus used as a food source in the snail rearing facility, were preferred resources, is difficult, especially since snails given these resources had significantly lower fecundity (see Strouse et al. 2021).

These results highlight the large knowledge gaps in defining the key food resources for Hawaiian arboreal snails. Such knowledge shortfalls are a major hindrance to our ability to conserve biodiversity effectively (Hortal et al. 2015). Here, we explore the utility of integrating field studies that examine which plants snails prefer and avoid as hosts with metagenomic analyses which might form the foundational first step to identifying important food resources for Hawaiian arboreal snails. Three recent studies have explored which plants native arboreal snails prefer as hosts in montane wet forests on three islands: Oahu, Maui, and Hawaii (Meyer 2012; Meyer et al. 2014, 2022). While this research was conducted on three separate islands and explored the preferences of many island endemic snails, concordant patterns emerged with snails across islands having relatively similar plant preferences (Meyer et al. 2022). Across islands and species, snails preferred a subset of understory plants. *Hydrangea arguta* was preferred by all snail species across all three islands. *Ilex* spp. and *Clermontia* spp. were preferred on Oahu and Maui, respectively, though *Ilex* spp. were avoided on Hawaii where non-native ginger (*Hedychium coronarium*) were abundant (Meyer 2012; Meyer et al. 2014,

2022). In contrast, snails avoided the two most abundant plants at all three sites: the dominant tree, *Metrosideros polymorpha*, and the mid-story ferns *Cibotium* spp. (Meyer 2012; Meyer et al. 2014, 2022). Using these results as a guiding framework, we employed a metagenomic approach to test the hypothesis that epiphytic fungal and bacterial assemblages differ between preferred and avoided plant species at Mt. Kaala, a site on Oahu where Meyer et al. (2014) previously examined plant preferences of arboreal snails. We also tested if relative abundances of *Cladosporium* spp. and *Botryosphaeria* spp. differ between preferred and avoid plant species. We chose these two fungi, because *Cladosporium* spp. has been used in the Hawaiian Tree Snail captive rearing facility and both fungi were preferred by snails in O'Rorke et al.'s (2016) preference study. Differences in bacterial and fungal richness, diversity, and evenness between preferred and non-preferred plant species were examined, but we had no *a priori* predictions about patterns of richness and diversity between preferred and avoided plants. If differences in microbial assemblages exist between preferred and avoided plant species, it provides a mechanism to: (1) develop hypotheses about which bacteria and fungi enhance arboreal snail survivorship and fitness, and (2) explore if concordant patterns with some microbes consistently having higher or lower abundances on preferred plant species exist across sites and islands.

Materials and methods

Sample collection

This study was conducted in the Mt. Kaala Natural Area Reserve (NAR), Honolulu, Hawaii (21.5064418°N, 158.1453868°W). The Mt. Kaala NAR is one of the few remaining intact, protected areas in the Hawaiian Islands. This site was chosen as arboreal snail plant preferences were previously described and the diverse flora on Mt. Kaala is composed primarily of native plants (Meyer et al. 2014). Studies in areas that harbor both native snails and are composed primarily of native plants are critical because high abundances of non-native species can modify snail behaviors and change the utility of the findings (Meyer et al. 2014, 2022). For example, identifying that snails prefer native plant species in areas that have lost significant native plant diversity may mean that sub-optimal plants are currently being used as hosts. Also, in sites where snails prefer non-native plants (see Meyer 2012), native plants with beneficial phyllospheres may be avoided, limiting restoration recommendations.

To test our hypothesis that phyllospheres differ between preferred and non-preferred plant species, we collected samples from five plant species: three plants preferred by snails, *Hydrangea arguta*, *Ilex anomala*, *Clermontia* sp.; and two plants avoided by snails, *Metrosideros polymorpha* and *Cibotium* spp. (Meyer 2012; Meyer et al. 2014, 2022). Sampling consisted of swabbing the stems, leaves (both top and bottom sides), and nodes of each plant with sterile cotton swabs which were placed in sterile 1.5 ml microcentrifuge tubes and stored in cold blocks (-20 °C) for transport back to the

Bishop Museum for processing. Swabs were collected from one individual of each plant species at three sites in July 2018 and seven sites in November 2020 in the Mt. Kaala NAR. *Clermontia* spp. samples were only collected in 2020. Sites are used loosely here as we collected samples from the closest individual to our stopping point and not within a defined area, but all sites were at least 100 m apart along a transect that followed the boardwalk through the NAR. Our goal was to collect samples from individuals that span multiple microhabitats within the Mt. Kaala NAR and to make sure that location was not responsible for differences in phyllospheres between preferred and avoided plants. All samples were stored in an ultracold (-80 °C) freezer prior to DNA extraction.

DNA extraction and sequencing

DNA was extracted with Qiagen's DNeasy PowerSoil Kit following the manufacturer's instructions with modifications for swabs. Briefly, all swabs were maintained in a cold block after removal from the -80 °C freezer and prior to being processed for extraction. Microbiome samples recovered on three swabs of each plant (node, stem, and leaf) were cut away from the swab using sterile scissors, being careful to minimize how much swab material was included. All three samples from each plant were combined in a single 2 ml tube containing Powerbeads and 60 µl of C1 solution provided with the kit. The microbiome cells were disrupted and lysed via homogenization in a Mini-Beadbeater 96 (BioSpec Products Inc., Bartlesville, OK) at 2600 rpm for 10 min. Following homogenization, tubes were centrifuged at 10,000× g for 30 s. Supernatant (~450 µl) was transferred to a clean, sterile 2 ml tube provided with the kit, and 250 µl of C2 solution were added to each. Samples were vortexed for 5 s and incubated at 4 °C for 5 min followed by centrifugation at 10,000× g for 1 min. Approximately 750 µl of supernatant was transferred to a clean 2 ml tube, and 1200 µl of c4 solution added to each tube. Samples were vortexed for 5 s to mix and 650 µl of the solution added to the MB Spin Column supplied with the kit. Samples were centrifuged at 10,000× g for 1 min to bind the DNA to the column and the flow through discarded. This was repeated twice with an additional 650 µl of sample each time until all the sample had been run through the column. The column filter was washed by adding 500 µl of C5 solution to each column and centrifuging at 10,000× g for 30 s. The flow through was discarded and the columns centrifuged an additional 1 min at 10,000× g. The column was placed into a clean 2 ml collection tube and 100 µl of C6 solution added to the filter. Extracted DNA was eluted via centrifugation at 10,000× g for 1 min. Eluted DNA was quantified using the Qubit 3 fluorometer and the high sensitivity DNA assay (Thermo Fisher, USA) and stored at -20 °C prior to sending to Molecular Research LP (https://www.mrdnalab.com/) for sequencing. Bacterial 16S DNA was amplified using primers 515F-Y and 926R from Parada et al. (2015), and fungal ITS2 regions were amplified using primers ITS1-F (Gardes and Bruns 1993) and ITS2 (White et al. 1990).

The 16S and ITS regions were amplified and sequenced by Molecular Research LP using the following protocol: The HotStarTaq Plus Master Mix Kit (Qiagen, USA)

was used for PCR with the following cycle: 5 min at 95 °C, then by 30 cycles of 30 s at 95 °C, 40 s at 53 °C and 1 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. Amplification success and relative quantity were verified via visualization on a 2% agarose gel. Samples were pooled in equal proportions using unique dual indices and purified using calibrated Ampure XP beads. The pooled and purified PCR products were used to create an Illumina DNA library. Sequencing was performed using Illumina MiSeq following manufacturer guidelines (Illumina, San Diego, CA, USA).

All sequences used in this paper are available at GenBank's SRA database under Bioproject PRJNA880325.

Bioinformatics

Reads were processed using Qiime2 (Bolyen et al. 2019). For fungi, all samples were imported into Qiime2 and the ITS1 region was extracted from each read using the Q2_ITSxpress plugin (Rivers et al. 2018). For the bacterial reads, we used the cutadapt Qiime2 plugin (Martin 2011) to filter reads without the primer sequences in the forward and reverse reads.

For both bacteria and fungi, the reads were processed using DADA2 (Callahan et al. 2016) (using the q2-dada2 qiime plugin) to generate a table of unique amplicon sequence variants (ASV) and their counts per sample. Taxonomy for each ASV was determined using the q2-feature-classifier plugin (Bokulich et al. 2018) classify-sklearn naïve Bayes taxonomy classifier against the Silva database version 138 for bacterial ASVs (Quast et al. 2013), and the Unite database version 8.3 for fungal ASVs (Abarenkov et al. 2021).

The bacterial samples were rarefied to 8,000 reads and the fungal samples to 19,000 reads based on the number of reads of the sample with fewer reads, and alpha and beta diversity measures were calculated using Qiime's diversity plugin. The high sequence variability of the ITS region makes multiple sequence alignment of this region highly unreliable for distantly related groups of fungi, which result in unreliable phylogenetic trees (Fouquier et al. 2016). Because of this, phylogenetic based measures of alpha and beta diversity are not recommended for ITS based fungal amplicon samples.

For each sample we calculated α-richness (number of different ASVs), diversity (Shannon Entropy), and evenness (Pielou Evenness index). For the bacterial samples we also calculated phylogenetic diversity (Faith's phylogenetic diversity metric). To estimate the differences between samples we used the Bray-Curtis index.

All the commands used in the Qiime2 analyses are available on the GitHub repository: https://github.com/aroc110/Meyer-et-al-2022.

Statistical analyses

To test if bacterial and fungal assemblages differed between preferred and avoided plants and among the five plant species, we ran four PERMONOVA tests. Bray-Curtis distance matrices for both bacteria and fungi assemblages were calculated in Qiime2

and were uploaded into PRIMER-E with the PERMONOVA+ add on (Clarke and Gorley 2006). First, we ran a two-factor PERMONVOA for both bacterial and fungal assemblages using plant preference (preferred and avoided) and sampling date (July 2018 and November 2020) as factors. Second, we ran a two-factor PERMONVOA using plant species and sampling date as factors for both bacteria and fungi. Following significant tests for plant species, we ran pairwise permutation-based t-tests to assess which plant species harbored different phyllosphere assemblages using a Bonferroni correction to account for multiple testing. Corrected α-values were 0.005 for the ten pairwise tests. We constructed MDS plots for both bacteria and fungi to visualize differences in microbial assemblages between preferred and avoided plants and among the five plant species.

We intended to test if relative abundances of fungal species (*Cladosporium* spp. and *Botryosphaeria* spp.) used in captive rearing efforts and found to be preferred fungi in lab feeding trials (O'Rorke et al. 2016; Strouse et al. 2021) differed on preferred and avoided plant species. However, because most samples did not contain either of these genera, we were not able to test the hypotheses that these two genera are more abundant on preferred plant species. Instead, we created box plots to show relative abundance of both genera between preferred and avoided species. Because median values for the relative abundances of both *Cladosporium* spp. and *Botryosphaeria* spp. on both preferred and avoided plants were zero, meaning that most samples did not contain sequences from either genus, we also reported the proportion of samples that contained each genus.

To test if fungal and bacterial ASV α-richness, diversity (Shannon), evenness (Pielou), and phylogenetic diversity (Faith) differed between preferred and avoided plant species and among the five plant species, we ran fourteen univariate PERMONOVAs using PRIMER-E with PERMANOVA+ add on. Faith phylogenetic diversity was not calculated for fungal samples as the ITS region used is well suited for distinguishing species, but it is too fast evolving to create reliable trees (Fouquier et al. 2016). Similarity matrices for each metric were created using the Euclidian similarity index. For each metric for bacteria and fungi, seven and six, two-factor PERMONOVAs were run, respectively. Half examined differences using preference status (preferred and avoided) and sampling date (July 2018 and November 2020) as factors, while the others used plant species and sampling date as factors. Following significant tests for plant species, we ran pairwise permutation-based t-test to assess which plant species harbored assemblages with higher richness, diversity, or evenness using a Bonferroni correction to account for multiple testing (α-values were 0.005 for the ten pairwise tests).

Results

Bacterial (F_1 = 3.51; P = 0.0001) and fungal (F_1 = 2.46; P = 0.0001) assemblages differed among preferred and avoided plant species (Fig. 1). While bacterial assemblages did not differ between sampling dates (F_1 = 1.26; P = 0.066), fungal assemblages

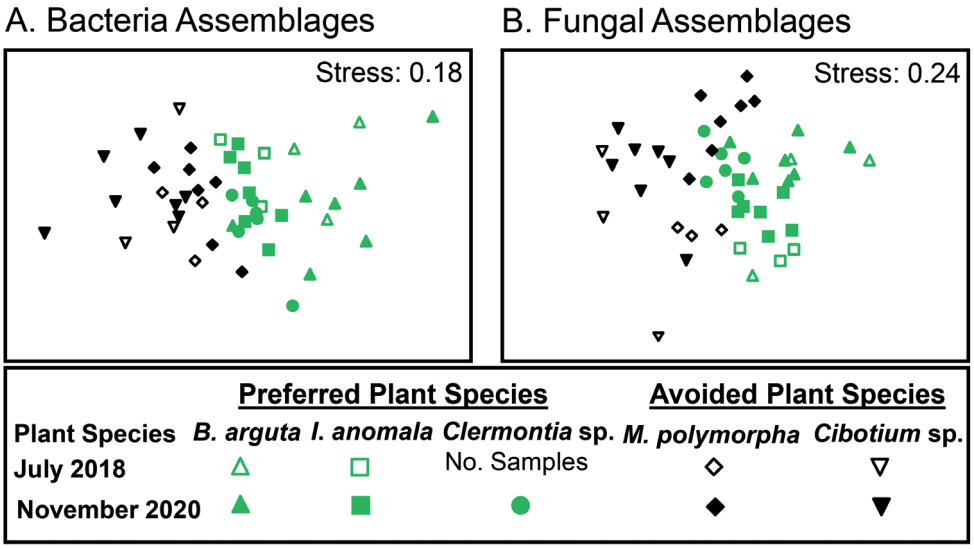

Figure 1. MDS ordination showing relationships between preferred and avoided plant species and among the five plant species sampled according to the composition and relative abundance of bacterial and fungal ASVs on each plant. Similarity was determined using the Bray–Curtis distance coefficient. Sites that are closer together are more similar in terms of ASV composition.

(F_1 = 1.46; P = 0.007) did. No significant plant preference by sampling date interactions were observed. We also found that bacterial (F_1 = 2.92; P = 0.0001) and fungal (F_1 = 2.46; P = 0.0001) assemblages differed among plant species (Fig. 1). When plant species was used as a factor, both bacterial (F_1 = 1.31; P = 0.030) and fungal (F_2 = 1.46; P = 0.006) assemblages differed between sampling dates. For analyses that explored differences among plant species, no significant plant species by sampling date interactions were observed. Pairwise comparisons revealed that all plant species harbored unique bacterial and fungal assemblages (Table 1).

We found that fungal taxa used in captive rearing efforts (*Cladosporium* spp.) and identified as potentially preferred taxa (*Cladosporium* spp. and *Botryosphaeria* spp.) in feeding trials (O'Rorke et al. 2016; Strouse et al. 2021) were present in less than 30% of the samples and that abundances of these two genera were generally low, although one sample contained > 10% *Cladosporium* spp. (Fig. 2).

Bacterial and fungal α-richness, evenness (Pielou), and diversity (Shannon) did not differ between preferred and avoided plant species (Fig. 3). However, preferred plant species had higher bacterial phylogenetic diversity than avoided plant species and phylogenetic diversity was higher on *Hydrangea arguta* than *Cibotium* spp. (Fig. 3). Differences in sampling date were only observed for fungal α-richness (analyses with preference categories, F_1 = 9.54; P = 0.0041; analyses examining differences among plant species, F_1 = 7.36; P = 0.0107) with fungal richness elevated in November 2022. Sampling date was non-significant for all other metrics examined.

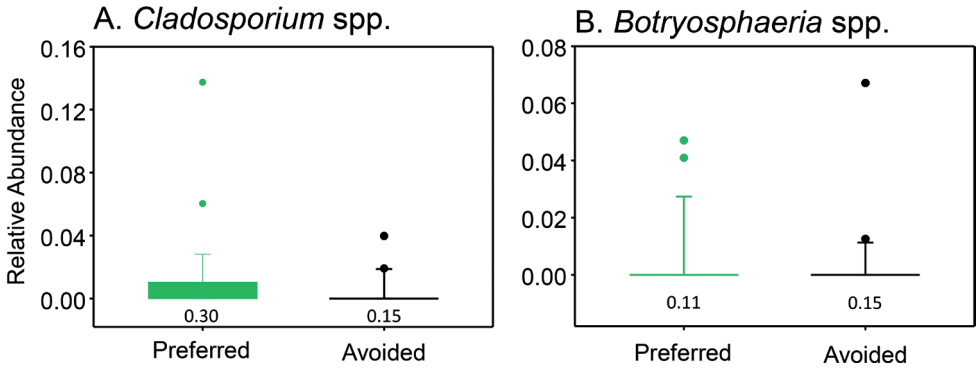

Figure 2. Relative abundances of *Cladosporium* spp., a fungus used in captive rearing facilities in Hawaii, and *Botryosphaeria* spp., a fungus preferred in lab feeding trials on preferred (green) and avoided (black) plant species. The numbers below each bar report the proportion of samples that contained each genus.

Table 1. Pairwise comparisons in fungal and bacterial assemblages among plant species. All pairwise comparisons were significant. Corrected α-values were 0.005 for the ten pairwise tests. ^{P} preferred plant species, ^{A} avoided plant species.

	Bacteria		Fungi	
Pairwise comparison	**t**	**$P_{(perm)}$**	**t**	**$P_{(perm)}$**
B. arguta^{P} vs. *Clermontia* sp.^{P}	1.53	0.0004	1.39	0.0006
B. arguta^{P} vs. *I. anomala*^{P}	1.77	0.0001	1.55	0.0001
B. arguta^{P} vs. *M. polymorpha*^{A}	1.87	0.0001	1.50	0.0001
B. arguta^{P} vs. *Cibotium* spp.^{A}	1.84	0.0001	1.64	0.0001
Clermontia sp.vs. *Ilex* spp.^{P}	1.34	0.0026	1.34	0.0031
Clermontia sp.^{P} vs. *M. polymorpha*^{A}	1.59	0.0002	1.28	0.0010
Clermontia sp.^{P} vs. *Cibotium* spp.^{A}	1.77	0.0001	1.56	0.0006
I. anomala^{P} vs. *M. polymorpha*^{A}	1.68	0.0001	1.50	0.0001
I. anomala^{P} vs. *Cibotium* spp.^{A}	1.87	0.0001	1.72	0.0001
M. polymorpha^{A} vs. *Cibotium* spp.^{A}	1.53	0.0001	1.50	0.0001

Discussion

Our approach, comparing phyllosphere assemblages between plants preferred and avoided by Hawaiian arboreal snails, may form a foundation to identify which microbes are key components of arboreal snail diets. For decades, it has been observed that Hawaiian arboreal snail distributions are patchy and that snails are often clustered on a few native plant species (Hadfield 1986; Meyer et al. 2014, 2022; Price et al. 2017). This specificity contrasts with the idea that arboreal land snails have been described as generalist grazers, consuming what is on leaf surfaces and not selecting specific bacteria or fungi (O'Rorke et al. 2015), and the idea that Hawaiian land snails represent a non-adaptive radiation (Rundell 2011). However, these observations are not mutually exclusive, as snails may be selecting plants with preferred phyllosphere assemblages. Unfortunately, previous research has not allowed us to test this hypothesis

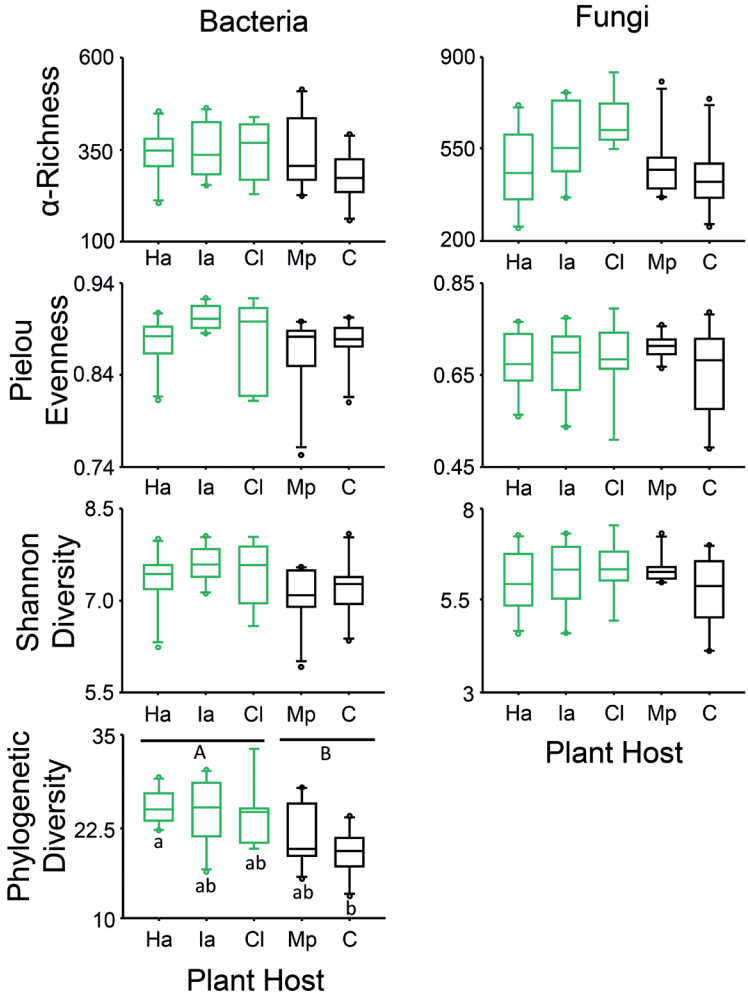

Figure 3. Fungal and bacterial ASV α-richness, diversity (Shannon), evenness (Pielou), and phylogenetic diversity (Faith) for preferred (green) and avoided (black) plants species and the five plant species sampled: Ha, *Hydrangea arguta*, Ia, *Ilex anomala*, Cl, *Clermontia* sp.; Mp, *Metrosideros polymorpha*; C, *Cibotium* spp. Capital letters with horizontal lines indicate differences between preferred and avoided plants and under case letters indicate differences among plant species.

as plants that did not serve as snail hosts were excluded from analyses (O'Rorke et al. 2015; Price et al. 2017). When designing this study, inclusion of avoided plant species as a comparison was identified as a key step, moving research away from characterizing which microbes snails encounter to testing if snails may be choosing plants based on their phyllosphere assemblage. The finding that preferred and avoided plant species have different phyllosphere assemblages indicates that Hawaiian arboreal snails are potentially selecting plants based on their phyllospheres, highlighting that their feeding behaviors are more nuanced than the classification as generalist grazers suggest.

Our approach is species (both plant and snail) and location specific. First, much of the confusion in synthesizing previous research lies in how we classify and choose which plant and snail species to study and where to conduct our studies. For example, Holland et al. (2017), using ginger (*Hedychium coronarium*) and jasmine (*Cestrum nocturnum*), concluded that egg production by *Auriculella diaphana* was reduced when snails grazed on non-native plant surfaces. In contrast, O'Rorke et al. (2015) found that phyllosphere assemblages on non-native strawberry guava (*Psidium cattleianum*) and coffee berry (*Schinus terebinthithifolius*) did not differ from native host plants. Consequently, classifying plants based on their native origin may have little utility in determining which plants harbor key phyllosphere resources. Second, it is important to realize that abiotic and biotic changes across sites impact phyllosphere assemblages (O'Rorke et al. 2015), meaning extrapolating snail preference patterns from one site to another may be inappropriate. Therefore, understanding how changes in conditions across sites impact phyllosphere assemblages on various plant species and how these changes influence preferences of different Hawaiian snails is key to developing informed conservation practices. For example, though reducing snail fitness for snails on Oahu, ginger (*H. coronarium*) was preferred by native succineids on the island of Hawaii (Brown et al. 2003, 2006; Meyer 2012; Holland et al. 2017). Understanding how phyllospheres differed between the two sites would help elucidate if differences were due to the various snail species being studied or because phyllospheres differed on ginger from these two sites. In addition to understanding how phyllospheres vary spatially, understanding how various abiotic conditions influence phyllospheres is important to predicting how a changing climate could influence these important resources and impact snail persistence.

We recognize that different arboreal snail species may require different phyllosphere resources. Previous research has primarily focused on protecting the remaining Hawaiian Oahu Tree Snails in the genus *Achatinella*, of which all 42 species are either extinct (33) or listed as endangered (9) (USFWS 1981). Observations and empirical evidence suggest that *Achatinella* spp. prefer ohia (*M. polymorpha*), a widespread native tree species, and that phyllospheres from this plant species enhance snail fitness (Hadfield 1986; Holland et al. 2017 Price et al. 2017; Sato et al. 2018; Strouse et al. 2021). In contrast, all snail species, none of which are *Achatinella* spp., in examined montane wet forests on Oahu, Maui, and Hawaii were found to avoid ohia (Meyer 2012; Meyer et al. 2014, 2022). Two scenarios are possible: (1) different conditions in montane wet forests mean that phyllospheres on ohia in these forests differ to the wet, but drier, forests in which *Achatinella* spp. are found, or (2) *Achatinella* spp. have evolved to use different phyllosphere resources from the snail species in the montane wet forests. Understanding the subtleties is important when collecting plant material for captive reared endangered snails, or when translocating snails to areas protected from predators, and when new snail species are added to conservation efforts (Sischo et al. 2016; Strouse et al. 2021).

It is also important to explore how phyllospheres differ among seasons and years. We found that fungal assemblages on preferred and avoided plants differed between

our two sampling dates, July 2018 and November 2020, but bacterial assemblages did not. When differences in phyllospheres among different plant species were examined, both fungal and bacterial assemblages differed among sampling date. These differences were slight relative to preference and species effects. Still, understanding seasonal and interannual variation, in concert with examining how phyllospheres differ across sites, can provide insights into how phyllospheres may change over various gradients and will allow us to predict how climate change may impact the remaining Hawaiian arboreal snails (Ovando et al. 2019; Teles et al. 2022). Our data provide baseline data for the Mt. Kaala NAR, and O'Rorke et al. (2015, 2017) and Price et al. (2017) provide valuable baseline data for other sites throughout Oahu. A concerted and collaborative effort by snail biologists and conservationists across the archipelago to identify long term study sites and explore how phyllosphere assemblages differ across sites and seasons/years using key plant species (maybe *M. polymorpha* for achatinellines, and *H. arguta* for other snails) and how changes in phyllospheres influence snail fecundity would enhance our ability to make informed conservation actions.

Knowing that preferred and avoided species have different phyllosphere assemblages allows us to develop hypotheses about which microbial species may be important snail food resources. However, we caution that this may be the most difficult step in the processes. Are abundant fungi and bacteria on preferred plants important for snail survival and fecundity, or are snails avoiding certain fungi and bacteria that may be pathogenic, less palatable, or reduce fitness? Hypothesis development is easy, but feeding trials like those conducted by O'Rorke et al. (2016) and experiments that examine differences in fitness like those conducted by Holland et al. (2017) and Strouse et al. (2021) are required to confirm that certain plants and microbial isolates enhance snail fitness. However, these trials need to incorporate taxa that are likely influencing preference and snail fitness. While *Cladosporium* spp. has been used in captive rearing facilities and in subsequent feeding experiments, we found that this genus is rare in most of our samples and has relatively low abundances on preferred and avoided plants in Mt. Kaala. Similarly, *Botryosphaeria* spp., a preferred fungus in O'Rorke et al.'s (2016) study was also rare and had low abundances on preferred and avoided plant species. Both *Cladosporium* spp. and *Botryosphaeria* spp. were chosen for lab rearing and feeding preference studies because they were able to isolate and grow these fungi in culture (O'Rorke et al. 2016). However, these species do not enhance snail fecundity or survivorship (Strouse et al. 2021). Because of this, lab rearing still includes collection of enormous amounts of live plant material which is not sustainable. We respect that isolation and growth of targeted fungi and bacteria are difficult, but we argue that randomly selecting fungi and bacteria for feeding trials using the criteria of which can easily be cultivated in a lab setting is a heuristic approach that may not yield key food resources and may not be in the best interests of effective snail conservation.

We also tested the hypotheses that snails are choosing plants with elevated richness and diversity. As we found no differences in both bacterial and fungal α-diversity, evenness, or diversity between preferred and avoided plants and among plant species, richness and diversity are probably not influencing snail preferences. However, for bac-

teria, we did find elevated phylogenetic diversity on preferred plants, suggesting that richness and diversity calculated using metrics that do not account for phylogenetic relationships among microbial taxa, may not adequately address the diversity of food resources snails encounter. While we could not run these analyses for fungi, we provide preliminary evidence that snails may also select plant species that host a more phylogenetically diverse assemblage of microbes.

Conclusions

We argue that our framework, which consists of first identifying which plants are preferred and avoided by arboreal snails, and then examining differences in phyllosphere assemblages between preferred and avoided plant species, is key to developing hypotheses about which microbes are important food resources for the remaining Hawaiian arboreal snails. Subsequent laboratory analyses would be required to determine if these microbial taxa influence snail fitness. If we could identify important microbes that form a healthy diet for Hawaiian arboreal snails, it greatly expands our ability to: (1) assess and monitor habitat quality by swabbing plants and assessing phyllosphere assemblages, (2) make informed restoration recommendations that may enhance arboreal snail survivorship and fitness, and (3) improve rearing efforts for highly endangered captive reared populations by enhancing survivorship and fitness and reducing the probability of introducing pathogens and toxins (Sischo et al. 2016). To develop effective long-term conservation practices, we also recommend that transformative long-term studies explore how snail preferences and phyllospheres differ across sites, seasons and years, and how changes in phyllospheres impact snail fecundity. The Hawaiian snail conservation community is extensive, with a large group of researchers and conservationists dedicated to protecting a unique snail fauna. This community has been working hard to stem the tide of snail extinctions for decades (Yeung and Hayes 2018), and recently has been coordinating efforts across labs to address some of the most pressing issues. We argue that conservation of Hawaiian arboreal snails is significantly hindered by our lack of knowledge about what they eat. We hope a coordinated effort using this framework can help elucidate key information that can help stem further extinctions.

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