Molecular assessment of wild
Achatinella mustelina diet
Final Report
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Introduction

*Achatinella mustelina* is a federally endangered tree snail endemic to the Waianae mountain range of Oahu. Efforts to exclude predators from native habitats combined with captive breeding programs have been reasonably successful at stabilizing small population sizes. Reproduction in captivity is inversely proportional with time in captivity, however, leading researchers to question diet adequacy. For this reason, we investigate the diet of wild snails. Because *Achatinella* snails are presumed to eat biofilms of fungi and bacteria off of leaf surfaces, we use molecular DNA barcoding methods to infer diet from deposited feces, since digested microbes are difficult to identify visually.

The first study of the project is now in press (Manuscript attached):


In this paper we investigate:

1) The diet of wild *Achatinella mustelina* in Oahu’s Waianae mountain range
2) Whether snail diet differs from leaf microbial composition (i.e. whether snails are “picky eaters”).
3) What environmental factors shape snail diet.

In addition to the project outlined above, we conducted a comparable analysis of how the diet of *A sowerbyana* differs over distance, precipitation and temperature in the Ko'olau range. The findings of this research were recently presented at the Island Biology 2014 meeting and a manuscript will be sent out to peer review by the time this report is submitted.

We have also cultured microbes that are significant in the diet of *A. mustelina*. These will be used in feeding trials to determine if the snails preferentially consume particular food items.

Samples

One hundred forty two field samples of *A. mustelina* feces, and the leaves from which they were obtained have been collected from field sites in the Waianae range: 36 from Puu Hapapa, 42 from Palikea, 18 from Kahanahai and 6 from Pahole. An additional 12 samples (leaves and feces) have been collected from Dr. Brendan Holland’s *A. mustelina* captive populations on UH campus. Two *Auriculella ambusta* (a non-endangered endemic tree snail) were also collected from Pu‘u Hapapa and dissected in order to compare gut contents with leaf surface and fecal microbial diversity.
Amend-Achatinella mustelina diet

Figure 1. Sampling locations on Oahu, Hawaii.

In general, multiple samples from each host plant species were sampled in each location in order to measure variance within and among host plants. A total of twenty-nine host plants were sampled including both native and non-native species.

**DNA Extraction and PCR Amplification**

Genomic DNA from both feces and leaves were extracted using a commercially available kit (MO-BIO PowerSoil). Feces were added to maceration tubes directly, whereas microbial biofilms from the leaf surface were sampled using sterile swabs. Both feces and leaf samples were PCR-amplified using DNA primers specific for Fungal (ITS1f and ITS4) and Bacterial (515f and 907r) barcode regions. Each sample was PCR-amplified using a short, unique “tag” sequence of nucleotides in order that sample identity is maintained throughout the analysis. These primers amplify neither plant nor animal DNA, so a positive result indicates successful amplification of target molecules. Negative control PCR/extraction reactions were run with sterile swabs that had not come into contact with the leaf surface. Absence of a positive PCR product from these “samples” indicates that target molecules derive from leaf surface/feces rather
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than lab contamination.

Table 1. Plant hosts sampled by location. Each sampling was a paired sample consisting of snail feces and a leaf surface swab.

<table>
<thead>
<tr>
<th>LOCATION</th>
<th>Elevation [m]</th>
</tr>
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<tbody>
<tr>
<td>Puu Hapapa</td>
<td>848</td>
</tr>
<tr>
<td>Palikea</td>
<td>897</td>
</tr>
<tr>
<td>Kahanahalani</td>
<td>671</td>
</tr>
<tr>
<td>Pahole</td>
<td>701</td>
</tr>
</tbody>
</table>

Results of Illumina Miseq Sequencing

Sequencing on the Illumina MiSEQ platform resulted in 13.8 million raw amplicon sequence reads in each sequencing direction. Overall read quality was excellent. Forward reads had mean phred scores >30 (that is >99.9% accuracy) for up to 270 of the 300 bp read length. Reverse reads were of poorer quality, having considerable variability in read quality after the 100th bp and mean quality dropped below a phred score of 30 by the 200th bp.

The long 300bp read length enabled by the new Miseq chemistry, means that there is a substantial amount of overlap between the forward and reverse reads. This enabled the construction of a consensus read with increased fidelity, and the present study yielded 12.8 million high quality consensus reads after pairing. Reads were finally assigned to their respective samples using golay error correcting barcodes. A total of 10.2 million high quality reads were successfully assigned to samples.
Figure 2. Mean Read Quality of Forward and Reverse Sequenced Amplicons from the Illumina Miseq run. Forward reads were consistently high quality through to the 280th base (phred>30). The mean quality of the reverse reads is still fairly high, although it drops off after the 200th base and has considerably more variability.
Analyses: what are snails eating and what factors structure their diet?

Are snails picky eaters? No.

Feces and leaf samples have equally species-rich microbial community assemblages and most fungal and bacterial reads that were sourced from leaves were also detected in fecal samples. However, the proportional abundance of these species differs between leaf and feces samples. This is indicated by analysis of a Bray Curtis transformation of the data, which shows that the dissimilarity between feces and leaf microbe diversity is a major component structuring the dataset (Figure 4).

To determine whether fecal composition does reflect gut composition – and is not the result of environmental contamination of feces, two Auriculella ambusta were dissected and their gut content compared to the leaf and feces samples. The result of this study was that 64% of reads were shared between the gut, leaf and feces, confirming that the phyllosphere is being passaged through the gut and detected in feces (Figure 6).

What factors structure snail diet and phyllosphere diversity?

The location of sampling and the taxonomic identity of the tree host are factors that structure the community composition of both fungus and bacteria. Samples from Pahole and Kahanahaiki are not dissimilar, but assort into a single
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Figure 4. Multi-dimensional scaling (MDS) plot of Fungal samples. Fungal community data was transformed into a Bray Curtis Dissimilarity matrix and visualized by MDS, which is an ordination approach to visualize dissimilarity. Points that are ranked most dissimilar are graphed furthest apart. Here there is a clear separation between the feces (fecal) and phyllosphere (leaf).

Figure 5. MDS plot with location highlighted as a factor. There is a clear gradient of dissimilarity indicating that location structures the microbial communities that constitute the diet of A. mustelina.

Other factors, such as whether the plant host is native or exotic don’t have a significant effect on variance. However, it must be observed that the only plants sampled in the present study were those that hosted snails. It cannot be ruled out that snails are either engineering the microbial community on which they feed, nor that snails select trees with certain microbial assemblages. Therefore, little
can be concluded about the component of variation explained by tree identity until a follow-up study is conducted to determine if the phyllosphere community structure is different between trees with snails and those without.

**What microbe are snails eating?**

Assigning a taxonomic identity to Species-level was problematic, likely due to high levels of endemism in Hawaiian microbes and a poor baseline database. Consequently, 86.5% of bacteria were identifiable at the order taxonomic level and 67.7% at the family level, compared to fungus, for which 63.4% taxa could be assigned an order and 47.3% a family. At the genus level, assignments were 38.4% for fungi and 32.61% for bacteria. In total, 7,376 fungal taxa and 3,967 bacteria taxa were detected. Microbial communities were very diverse and less than 50 species of fungi or bacteria contributed more than 0.3% of total sequence reads. The taxonomy of the most abundant fungal reads revealed that they were from fungal groups commonly associated with the phyllosphere, whereas bacterial OTUs were assigned to clades that are either common members of the phyllosphere or gut bacteria.

**Figure 6. Comparison of fungal read abundances in snail gut, feces and the phyllosphere.** Numbers represent proportions of total reads attributed to each component.
Comparisons between the *in situ* and captive snails diets.

A study was conducted to evaluate the diet of snails cultured in the *ex situ* facility (Brendan Holland’s lab on UHM campus) and how this compared their diet to wild snails.

Whereas the wild diet is highly diverse the captive snail diet is dominated by a single taxon. This taxon is the *Cladosporium* species (Order: Capnodiales) that is used as a dietary supplement *ex situ* raised snails. However, this also present in the wild diet and although nowhere near as dominant as it is in the *ex situ* facility it is a major component of the wild fungal assemblage, comprising approximately 2% of wild snail diets (Figure 7).

![Fungal assemblage from wild snails](image1)

**Figure 7. Rank abundance of fungal taxa from a) wild and the b) *ex situ* culture facility.** While wild populations of *Achatinella* have a diverse diet with no diet items dominating their gut content, the snails in the *ex situ* facility have a diet that is dominated by a single *Cladosporium* (pictured and highlighted red), which took up almost 40% of the sequenced reads from the feces of cultured snails. This taxon also occurred in the wild (highlighted in red), but occurred in abundances slightly less than 2%. Operational Taxonomic Unit (OTU) is a technical term meaning a molecular defined “species” equivalent.
Fungus and bacteria isolation from feces and leaves

We have begun isolating the microbes that are significant in the diet of *A. mustelina*. Ninety-two fungal and bacterial strains were isolated from leaves and snail feces sampled from trees at the Pu‘u Hapapa and Palikea enclosures. This work has confirmed that fungi are viable when they pass through the gut of snails and provides a set of potential food items that can be used to enhance captive culture of snails.

Feeding trials

The results of our study indicate that *A. mustelina* indiscriminately consume microbes from whatever surface they are on. However, this does not answer whether they have particular feeding preferences. To determine if they have feeding preferences we have prepared multiple microbial lawns that will be used to trial feeding preferences of *Auriculella ambusta*, a non-listed native tree snail in the same family that will be used as a surrogate for *Achatinella*. Our amplicon sequencing dataset shows that sympatric *Auriculella* and *Achatinella* consume similar microbes, which justifies this comparison. The trial apparatus has been assembled, which consists of 10 chambers into which individual snails will be placed. Stop motion photography will capture an image every 20 seconds over 24 hours (12 dark and 12 light) to determine which of 12 food choices the snails elect to eat. The permits for this experiment have been successfully obtained and trials will begin soon. A preliminary run of the experiment can be viewed at https://www.youtube.com/channel/UCBL4mGFBZsnfQQAw-ZDT1iQ.
Comparisons with congeners *Achatinella lila* and *Achatinella sowerbiana*

A separate, related study was conducted on the diet of two species closely related *A. mustelina*: *A. lila* and *A. sowerbiana* that co-occurring in the Koolau range. This manuscript is in advance preparation: Melissa R. Price, Richard O’Rorke, Anthony S. Amend, Michael G. Hadfield (In Prep) A Buffet Strategy for Grazing Hawaiian Tree Snails, Despite Geographic Structuring of Phyllosphere Fungi.

This study found that, similar to *A mustelina*, the snail diet does not differ from the microbial community of the leaves. There was also no evidence that diet differed between the two species with overlapping ranges (Table 2).

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>MS</th>
<th>Pseudo-F</th>
<th>P(perm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail Species</td>
<td>3865</td>
<td>3865</td>
<td>1.06</td>
<td>0.293</td>
</tr>
<tr>
<td>Fecal or Leaf</td>
<td>11854</td>
<td>11854</td>
<td>3.25</td>
<td>0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>1.28E+05</td>
<td>3652</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1.44E+05</td>
<td></td>
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However, fungal communities found in snail feces were much more similar to each other than were communities found on leaf surfaces, supporting the hypothesis that snails may exert top-down selection on phyllosphere communities by selective digestion (Figure 9).
Conclusions

Although *A. mustelina* subsists entirely by grazing microbes from leaf surfaces of native trees, little was known about fundamental aspects of these microbe assemblages: not taxonomic composition, how this varies with host-plant or location, nor whether snails selectively consume microbes. To address these questions we have completed a high-throughput MiSeq sequencing study to determine how microbial community structure varies across the habitat range of the endangered tree snail *A. mustelina*. We have found that:

- Snails are not selective feeders, but are generalists at the phyllosphere scale
- The diversity of the microbial communities that snails feed on is surprisingly high, with few species dominating the assemblage
- By contrast, the diet of captive raised snails is dominated by a single taxon
- Microbes consumed by snails vary by location and host-plant identity, with fungal community composition being strongly determined by location
**Future work: determining the implications for management**

Snail conservation is currently dependent on the success of *in situ* field enclosures. These enclosures are situated in the natural ranges of the snails, and therefore they do not need to simulate the natural environment. However, the results of our present work raise an important question: can we simulate the microbial communities that snails live on in the laboratory or in convenient and accessible patches of land? If this were possible, then snail enclosures could be built near conservancy offices, eliminating the expense, danger and climatic unpredictability associated with building enclosures in the field and transporting the materials to these enclosures. It would also eliminate the need to collect leaves for the captive snails from the wild. This might eliminate expense, and eliminate a potential vector for disease.

We will next undertake an experiment to determine if domesticated trees can be inoculated with a 'wild' microbial assemblage. This will be complimented by a set of experiments that will determine what the minimal requirements are for maintaining this assemblage *ex situ*. The results of our present study lead us to devise a set research objectives for the coming year that will help with snail management:

- Can we simulate a wild snail diet?
- How does snail translocation affect the composition of the microbial community?
- What are the affects of sourcing leaves from multiple locations for *ex situ* propagation?
- What are the impacts of the lack of dietary diversity in captive cultures?
- The tools developed in the present study should be used to determine differences between sites colonized by snails and sites that are not.
Environmental Microbiology

Dining local: the microbial diet of a snail that grazes microbial communities is geographically structured

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Subject category: Microbe-microbe and microbe-host interactions

Running title: Diet of A. mustelina
Abstract

*Achatinella mustelina* is a critically endangered tree snail that subsists entirely by grazing microbes from leaf surfaces of native trees. Little is known about fundamental aspects of these microbe assemblages: not taxonomic composition, how this varies with host-plant or location, nor whether snails selectively consume microbes. To address these questions we collected 102 snail fecal samples as a proxy for diet, and 102 matched-leaf samples from four locations. We used Illumina amplicon sequencing to determine bacterial and fungal community composition. Microbial community structure was significantly distinct between snail feces and leaf-samples, but the same microbes occurred in both. We conclude snails are not ‘picky’ eaters at the microbial level, but graze the surface of whatever plant they are on. In a second experiment, the gut was dissected from non-endangered native tree snails in the same family as *Achatinella* to confirm that fecal samples reflect gut contents. Over 60% of fungal reads were shared between feces, gut and leaf samples. Overall, location, sample type (feces or leaf) and host-plant identity all significantly explained the community composition and variation among samples. Understanding the microbial ecology of microbes grazed by tree snails enables effective management when conservation requires captive breeding or field relocation.

**Key words:** Phyllosphere/Microbial community/Diet/MiSeq/Beta-diversity/tree snail

Introduction
The Hawaiian tree snail genus *Achatinella* once comprised approximately forty-one species, all restricted to narrow ranges, endemic to the island of Oahu. Although considered extremely rare today, as recently as the 19th and early 20th centuries the Hawaiian tree snails were abundant, broadly distributed and could be viewed by the thousands during a single excursion in the forest. The combined pressures of shell collection, predation by alien species (Chiaverano *et al.* 2014; Holland *et al.* 2012) and habitat alteration and loss have severely impacted achatinelline species diversity and distributions. Today, only ten species remain in the genus *Achatinella* (Holland & Cowie, 2009), all of which are listed as federally endangered (USFWS 1981) and require extensive conservation intervention to prevent the extinction of remaining species. *Achatinella mustelina* is endemic to the Waianae mountain range of western Oahu, and consists of six genetically distinct populations (Holland & Hadfield, 2002; 2007). However, the snails are patchily distributed and it is unknown if their diet is similar across the distribution. All members of the Hawaiian tree snail subfamily Achatinellinae feed on microbial communities growing on the surface of (predominantly native) leaves, but attempts to characterize their microbial diet have been limited to the isolation of a single *Cladosporium* sp. fungus, isolated from a native Ohia tree (*Metrosideros polymorpha*), for the purpose of *ex situ* tree snail culture (Kobayashi & Hadfield, 1996). The objective of the present study was to examine how geography and host-plant taxonomy determines the microbial diet of this rare endemic Hawaiian tree snail.

Recent applications of molecular methods to characterize the diet of consumers via the digesta (Pompanon *et al.*, 2012) hold potential to expedite our
understanding of fundamental aspects of diet ecology and feeding behavior in the wild. DNA based approaches have been successfully applied to animals that consume fungus (Jørgensen et al., 2005; Remén et al., 2010; O'Rorke et al., 2013; Soininen et al., 2013). A cloning-based study showed that all 144 fungal DNA reads taken from the guts of several collembolans were Aspergillus, excluding a range of potential diet items (Jørgensen et al., 2005). A pyrosequencing study found that the fungus in the guts of Norwegian lemmings derived predominantly from species with small fruiting bodies and concluded that it was unlikely that mycophagy plays a major role in the mostly herbivorous diet (Soininen et al., 2013). However, these studies involved the sacrifice of the consumer in order to access their gut contents, and are therefore unsuitable models for the endangered A. mustelina. A non-lethal approach to DNA diet studies is to extract DNA from the feces, which has been demonstrated with large herbivores and carnivorous predators (Barnett et al., 2010; Oehm et al., 2011; Parsons et al., 2005; Brown et al., 2011; Deagle et al., 2009).

Comparatively more is known about the microbes comprising the phyllosphere in general. This is due to a steady effort of research based on culture and characterization of microbial isolates (e.g. Morris et al., 1998; Baker et al., 1979), and molecular characterization of the phyllosphere (e.g. Knief et al., 2010; Thompson et al., 1993). More recently, there has been a surge in community-level information about the phyllosphere facilitated by the high yields of sequence data from high-throughput DNA sequencing technologies (Jumpponen & Jones, 2009; reviewed in Müller & Ruppel, 2013; Vorholt, 2012), suggesting that the phyllosphere is structured primarily by distance, local environment and
host-plant characteristics. Importantly, these more recent studies have moved away from model systems and are using the power of high-throughput technologies to explore phyllospheres in wild plant assemblages (Kembel & Mueller, 2014; Kim et al., 2012; Finkel et al., 2011).

For the present study we assessed the variance in community composition of the microbes constituting the diet of *A. mustelina*, a federally endangered species. We sequenced DNA from *A. mustelina* feces occurring on multiple replicate host trees from four sites in the Waianae mountain range, to measure the extent to which location and plant host identity correlate with diet. Paired samples from leaf microbial communities were also collected, enabling us to assess whether microbial community variance in feces was correlated with that of the phyllosphere. To evaluate the extent to which microbes detected in the feces correspond with gut content, we also dissected the gastrointestinal tract from two *Auriculella ambusta* individuals, which are non-endangered snails in the same family as *A. mustelina*, and compared the microbial composition to that of their feces and the phyllosphere. Finally, we discuss how novel molecular methods within a tri-trophic ecological framework can abet basic natural history research for the conservation of an enigmatic endangered species.

**Methods**

**Sampling**

Sampling stations and dates are given in table 1. To ensure the sampled feces were recently deposited, tree foliage was searched at nighttime for snails, and the trees in which snails were present were flagged. *Achatinella* feces are
distinctively large and any that were freshly deposited on leaf surfaces of the
flagged trees were then collected the subsequent morning. Efforts were made to
locate snail feces on a range of host-plant species at each site. Snail feces were
transferred from leaves into sterile microcentrifuge tubes using sterile forceps.
Leaf microbes were sampled by applying a sterile swab to both leaf surfaces.
Two *Auriculella ambusta* snails were also collected from the Puu Hapapapa
station (Figure 1) and transported along with host leaves in a container so that
the gut could be dissected under laboratory conditions.

*DNA extraction, PCR amplification, and sequencing*

Genomic DNA from feces, leaf swabs and the *Auriculella ambusta* gut were
extracted using a commercially available kit (MO-BIO PowerSoil) following the
manufacturer’s instructions. Negative control PCR/extraction reactions were run
with sterile swabs that had not come into contact with the leaf surface.

PCR reactions were run in duplicate and targeted the nuclear ITS1 region of
fungus as well as the V4 region of the bacterial 16S rRNA using thermal cycler
programs and PCR primers as in Smith and Peay (2014). All samples were PCR
amplified using fusion primers that consisted of a locus specific priming site at
the 3’ end, the ‘a’ or ‘b’ Illumina adapter at the 5’ end and, in the case of the a-
adapter primer, an error-correcting Golay barcode in between (Caporaso *et al.*, 2011). PCRs were conducted in 25 µl reactions using 1× Phusion® Hot Start Flex
mix (New England Biolabs, Massachusetts), primer A (0.2 µM), primer B (0.192
µM) and gDNA (~5 ng). PCR products were visualized on 1.25% agarose gels and
duplicate positive reactions were combined and made equimolar using
SequalPrep™ Normalization plates (Invitrogen, New York). All fungi and bacteria libraries were subsequently pooled, cleaned using a SPRI plate (Beckman Coulter, California) and Sera-Mag Speed-beads (FisherSci, Pittsburgh) in an amplicon:bead ratio of 1.8:1, and quantified on a Qubit fluorometer (Invitrogen) using the dsDNA HS assay. Fungi and bacteria amplicons were then combined in a 3:2 ratio (as per the recommendation of; Smith & Peay, 2014) and were finally subject to quality control on a Bioanalyzer Expert 2100 High Sensitivity chip (Agilent Technologies, California) and qPCR to determine cluster density before sequencing.

Sequencing was undertaken at the University of Hawaii, Genetics Core Facility using the Illumina MiSeq platform with the MiSeq Reagent v3 chemistry (Illumina) that enables 300bp paired-end reads. Three primers were used for each amplicon sequenced, one for each sequencing direction and for the sample index ID. Raw paired end reads are available in NCBI’s SRA under accessions SAMN03019997 - SAMN03020200.

**Sequence processing**

Full processing pipeline commands are available online in the supplementary material, but briefly: FASTQ files were quality checked (S2) and forward and reverse reads merged (PEAR; Zhang et al., 2013). Paired reads were assigned to samples (Caporaso et al., 2010) and then dereplicated, chimera screened (UCHIME; Edgar et al., 2011) and clustered at 97% (UPARSE; Edgar, 2013). Taxonomy was assigned using the Wang method (MOTHUR; Schloss et al., 2009) against either the Greengenes database (for bacteria) or a modified UNITE
database for fungi augmented with non-target outgroup taxa (refer to Supplemental Scripts).

**Data analyses**

Apart from the mixed-effects model analyses, which were conducted in PRIMER-6 (Clarke & Warwick, 2005), data were analyzed in R using the packages vegan (Dixon, 2009), MASS (Venables & Ripley, 2002), indicspecies (De Cáceres & Legendre, 2009) and were visualized with ggplot2 (Wickham, 2009). Full scripts are available in the supplementary materials. Libraries were rarefied to 2,000 reads and samples with less than 2,000 reads were discarded. Samples were square root transformed to down-weight the influence of excessively abundant reads and used to calculate a Bray-Curtis dissimilarity matrix (Bray & Curtis, 1957). Correlation between fecal and leaf matrices were assessed using a Mantel test (Mantel, 1967). The influence of different factors on variance were then visualized by NMDS ordinations and modeled using PERMANOVA (Anderson, 2005) under a reduced model. Due to the imbalance of some of the sampling levels, a type III (partial) sums of squares was used. Individual OTUs that were significantly associated with a particular factor were subsequently identified using indicspecies (De Cáceres & Legendre, 2009).

**Results**

**Sampling results**

A total of 102 snail feces/phyllosphere pairs were sampled from Puu Hapapa, Palikea, and from adjacent sites at Kahanahaiki and Pahole (Table 1). The snails were collected from a diverse range of host-plant species (Table 1) including the
exotic species *Psidium cattleianum* (strawberry guava) and *Schinus terebinthifolius* (Christmas berry) sampled because they periodically serve as non-native tree snail host-plants. A fecal/phyllosphere pair was also collected from plastic flagging tape, on which snails are occasionally found.

Sequencing results

The mean number of fungal reads (± sd) was 18,777 ± 568 per sample and for bacteria reads was 9,435 ± 303. Four fungal samples and eleven bacterial samples had < 2,000 reads and were removed from subsequent analysis. Total richness was high with 7,376 fungal OTUs and 3,967 bacteria OTUs being detected after removal of singletons and rarefaction of samples. Microbial communities followed a log-normal distribution with a long tail with only 10 fungal and 12 bacterial OTUs contributing more than 1% of total reads (Figures 2a and 2b). The β diversity between all samples was highly diverse (Bray-Curtis dissimilarity ± sd: fungi = 0.85 ± 0.07, bacteria = 0.73 ± 0.09).

Taxonomic assignment of OTUs

At higher taxonomic resolutions the bacteria OTUs are mostly identifiable using our methods, with 86.5% of bacteria assigned to an order and 67.7% a family, compared to fungus, for which 63.4% OTUs could be assigned an order and 47.3% a family. At taxonomic scales such as genus, assignments were at 38.4% for fungal and 32.61% for bacterial reads.

Factors that structure microbial beta diversity
The community composition of microbes was structured by sample type (feces/leaf), geographic locations from which samples were taken, and taxonomic identity of the tree host (Table 2). Samples taken from the host plant *Mersine lessertiana*, which occurs across the three major sampling locations, were also analysed and both geographic location and sample type remained significant factors (p<<0.5) that explained sample variance in both bacteria and fungi (Supplementary tables 1a and 1b). Furthermore, when analyses are constrained to the three plant host orders that were abundant across all sites it is the case that geographic location, sample type and plant host order remain significant factors, and geographic location remains the greatest factor that determines variance (Supplementary tables 1c and 1d). Whether leaves were from native or exotic host-plants was not significant (p>0.05). For fungi, location explained a greater component of variation than the other factors (Figure 4). By contrast, location, host-plant and sample type were largely equal components of variation for bacteria communities (Figure 4).

*Does fecal assemblage resemble leaf assemblage?*

To determine whether snails were selective in their diet we used indicator species analysis and identified that no fungal OTUs were statistically associated with leaves and two OTUs (order Chaetothyriales) were associated with feces and not leaves (Table 3). However, there were no OTUs that occurred exclusively on either feces or leaves. The β diversity of fungal OTUs detected in feces correlates positively with that of the phyllosphere (Mantel test, r = 0.58, p=0.001) suggesting that both are likely to be structured by similar determinants. The β diversity of bacteria leaf and fecal OTU assemblages are also
positively correlated, although to a lesser extent (Mantel test, $r = 0.41$, $p=0.001$) and do have OTUs that are significantly associated with either leaves (orders: Actinomycetales, Cytophagales and Saprospirales) or feces (Acidobacteriales, Enterobacteriales, Chthoniobacterales; Table 3b). Bacterial OTU_2 (Enterobacteriaceae) was the second most abundant bacterial OTU detected in the entire dataset (Figure 2), has a likelihood of 93% of occurring in feces, but only a 15% chance of occurring in the phyllosphere (Table 3).

Dissection experiment

Because snail feces are nutrient rich and moist, they likely provide an attractive substrate for environmental microbes. To determine the extent to which fecal microbial composition reflects gut composition, as opposed to exogenous colonization, two Auriculella ambusta were dissected and their gut contents compared to the leaf and fecal samples. Our dissection experiment showed that the majority of fungal sequencing reads (>60%) were shared between the gut, leaf and feces, supporting the hypothesis that these phyllosphere microbes are passaged through the gut and detected in feces (Figure 5).

Discussion

What determines microbial composition of Achatinella diet?

Achatinella mustelina has the largest natural distribution (~24 km; Holland & Hadfield, 2002) within the genus, and the strongest determinant of fungal and bacteria community composition was geographic location within the snail’s range (Table 2). As has become the de facto rule for microbial biogeography in general (Hanson et al., 2012), both geographic distance and environment have
been shown to play a role in structuring Hawaiian phyllosphere microbes. Our results are concordant with previous studies using both culture-based techniques (Baker et al., 1979; Marsh, 1966), and culture-independent techniques (Zimmerman & Vitousek, 2012), to demonstrate geographic patterning of microbial communities across spatial gradients in the Hawaiian Islands.

Whereas previous studies of the Hawaiian phyllosphere considered a maximum of three host species (Baker et al., 1979; Marsh, 1966, Zimmerman & Vitousek, 2012), we include measures of among-species variance here reflecting the diverse range of A. mustelina host-plants. Host-plants vary in morphology and biochemistry, and are therefore a selective substrate in the composition of microbial communities (Whipps et al., 2008). In other studies, host ecotypes have been shown to affect community composition more powerfully than geographic location (Cordier et al., 2012), which is a pattern that extends up to a global scale (Redford et al., 2010). A study of the tropical phyllosphere on Barro Colorado Island found that host-plant identity explained 56% of the variance among hosts of fungal epiphytes (Kembel & Mueller, 2014). However, although significant, host-plant identity was not the strongest explanatory factor in our study. This might be a property of the microbes that are abundant on the leaves of plants on which A. mustelina feed. For example Methylobacterium, which were a dominant bacterium in this study, have been shown to be structured by location more so than host identity and form similar communities across sympatric but unrelated host-plant species (Knief et al., 2010). Alternatively, the
snails might have a homogenizing effect on the phyllosphere community (discussed below).

*Does snail fecal composition correlate with phyllosphere composition?*

One objective of the present study was to assess the extent to which the microbial composition of snail feces resembled that of the phyllosphere in which the snails occurred, in order to determine if the snails were indiscriminately feeding on microbes or were feeding selectively. Almost all OTUs detected in the phyllosphere were also detected in feces, and only a few OTUs were indicators for feces or phyllosphere communities (Table 3). Furthermore, the dissection experiment of *Auriculella ambusta* confirmed that OTUs detected in the feces and phyllosphere were also found within the gut. In contrast to patterns observed with other consumers of hyphae (Jørgensen *et al.*, 2005), here there is no evidence that *A. mustelina* specialize in consuming any particular fungal or bacterial species present on leaf surfaces, and they can instead be considered true generalists that consume a wide range of microbes.

Our results suggest that snails are not ‘picky eaters’. The indicator species analyses found no microbes that were exclusive to either feces or leaves and that there were few microbes that were more likely to be associated with either feces or leaves (Table 3). There is also considerable overlap in microbe identity found in gut contents, feces and leaves (Figure 5). However, the community frequency distributions also appear to be skewed by the digestive process (Figure 3 and Table 2). The extent to which dietary composition is preserved through digestion has been investigated with predators of large multicellular organisms, and while
the diet’s composition remains constant throughout digestion for some predators (Bowles et al., 2011; Murray et al., 2011) this is often not the case (Deagle et al., 2013; 2010). Therefore, with a generalist consumer of complex microbial communities such as *A. mustelina* it is unlikely that the community frequency distribution will be maintained through the process of digestion.

**Diversity and Composition of Microbes in feces and phyllosphere**

The diversity of the microbial environment in which *A. mustelina* occurs is concordant with other recent studies of the tropical phyllosphere. The estimated (rarefaction) number of OTUs for fungal communities, 274 ±6, is slightly greater than that detected in non-surface sterilized leaves of *Metrosideros polymorpha* on Hawai’i Island which varied from 223 to 258 OTUs per rarefied sample (Zimmerman & Vitousek, 2012), and comparable to the Barro Colorado phyllosphere study: 279 ±6 (excluding OTUs with <10 reads; (Kembel & Mueller, 2014). The OTU distributions of both bacteria and fungi conformed to log normal distributions (Figure 2a, 2b), although bacterial distributions were characterized by greater dominance by fewer OTUs, which has been observed in a previous study of co-occurring soil fungi and bacteria (Hartmann et al., 2012).

The most common fungal classes detected in the present study were the Dothidiomycetes, Eurotiomycetes and Sordariomycetes, which is consistent with other investigations of the fungal phyllosphere (Kembel & Mueller, 2014). Fungi from the order Xylariales were also ubiquitous, with three OTUs from the genus *Pestalotiopsis* totalling 6.87% of reads (OTUs 3, 5, 6). *Pestalotiopsis* species and many of the other fungi that were discovered, such as *Khuskia*, are plant
specialists and are common plant endophytes in the tropics (Kembel & Mueller, 2014; Baker et al., 1979), but other abundant OTUs, such as OTU_1 (2.01%), assign to highly cosmopolitan and saprobic taxa such as Cladosporium (Bensch et al., 2010). While many of these OTUs can be identified as taxa that are commonly observed in the phyllosphere, there remain a considerable number of unidentified OTUs. This lack of taxonomic resolution points towards large geographic gaps in mycological research in Oceania and contrasts with previous inferences from culture-based studies that the Hawaiian phyllosphere consists of globally cosmopolitan species (Baker et al., 1979).

Several bacterial OTUs were observed across all samples. Many of these OTUs were from the subphylum Gammaproteobacteria (OTU_1, Oceanospirillales; OTU_1050, Enterobacteriae and OTU_4, Alteromonadales), which comprised 17% of reads. Of the Gammaproteobacteria the Enterobacteriales (e.g. OTU_2) were most abundant in fecal samples (Table 3), but these OTUs were also discovered on leaf surfaces. It is not unusual to find Enterobacteriaceae species in the phyllosphere (Hunter et al., 2010; Lopez-Velasco et al., 2011), which can be fairly widely distributed (Redford & Fierer, 2009) and the present study highlights the role that small invertebrates might play in occurrences of these bacteria. The Rhizobiales were ubiquitous in the present study and are frequently observed in the phyllosphere (Redford et al., 2010; Delmotte et al., 2009); some can fix nitrogen (e.g. Beijerinckia) and many can metabolise C₁ molecules (e.g. Beijerinckia and Methylobacteriaceae), which are a product of plant growth metabolism (Kutschera, 2007). The Alphaproteobacteria were also significant components of the phyllosphere and fecal samples. For example,
OTUs 12 and 258 from the diverse order Sphingomonadales, known for the ability to metabolise complex polymers, were also ubiquitous (Redford et al., 2010; Delmotte et al., 2009). Therefore, although the present study does not corroborate Baker et al. (Baker et al., 1979) in their inference the Hawaiian phyllosphere consists of cosmopolitan microbes, it does agree that the Hawaiian phyllosphere consists of functional groups of fungi and bacteria that commonly occur throughout the phyllosphere at a global scale.

Presence of tree snails: a common factor that determines microbe communities, a direction for future research

Animals, including snails, have been shown to alter the community structure of microbes on which they feed (Poulsen & Boomsma, 2005; Rollins et al., 2001; Aizenberg-Gershtein et al., 2013; Silliman & Newell, 2011; Sieg et al., 2013).

Snails rasping the surface of leaves can influence biosphere community succession and nutrient cycling, and may alter antimicrobial barriers of plants such as the wax layer and antimicrobial compounds therein (Yadav et al., 2005; Lindow & Brandl, 2003). Mucus secreted during terrestrial gastropod locomotion, know as the slime trail, has been shown to have selective antimicrobial properties (Kubota et al., 1985; Iguchi et al., 1985; 1982). A third potentially important factor in determining arboreal microbial community structure could be that tree snail feces recycle, deposit, and fertilize fungal spores back into the phyllosphere. This process may play an as yet undocumented role in determination and maintenance of microbial community structure. The fungal phyllosphere is presumably horizontally inherited (Osono
and tree snails could be a significant component in the transfer of fungus to recently budded leaves.

Achatinelline tree snails are frequently observed clustered on native host-plants, and only rarely observed on exotic species (Hadfield, 1986). These associations are not readily explained by plant traits per se, since host-plants vary widely in terms of stature, chemistry, and leaf surface characteristics, spanning multiple taxonomic classes. For this reason, it had long been hypothesized that phyllosphere microbial community composition would differ among host-plants that had evolved in Hawaii over millions of years, and those introduced within the last century. However, surface swabs and snail feces sampled from exotic host-plant taxa, and even plastic flagging tape, did not differ significantly. Plants that do not serve as snail hosts were not sampled in this study, so inferences about the role of snails in structuring their microbial environment may be a fruitful direction for future research.

Conservation implications

Although Hawaiian tree snails had been known to feed on microbes, the composition of these microbial communities had previously not been characterized. Determining the identity and distribution of the most abundant microbe lineages therefore provides the first baseline data for monitoring changes in the food-web structure of A. mustelina and provides information regarding candidate species of fungi and bacteria that might be isolated to complement existing efforts to safeguard snails via ex situ propagation (Kobayashi & Hadfield, 1996). The affects of abrupt diet changes on the
immediate health and long-term fitness of *A. mustelina* is an area that warrants further research, but the present study offers an effective approach to understand the composition of their microbial diet.

**Conclusions**

The present study used high-throughput MiSeq sequencing to determine if microbial community structure varies across the habitat range of the endangered tree snail *A. mustelina*. By comparing fecal samples to matched leaf samples we also addressed whether snails are selective feeders, and found that this species tends to be a generalist feeder, and that the microbes consumed vary with location and host-plant identity. The current method of safeguarding these snails against extinction is to relocate them to predator-proof enclosures in concert with *ex-situ* breeding. The outcomes of these conservation strategies are considerably enhanced through having determined the composition of the microbial communities the snails depend upon in their native habitat.
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Supplementary data, including scripts used to demultiplex, cluster and analyze data are available online.
References


Holland BS, Hadfield MG. (2002). Islands within an island: phylogeography and conservation genetics of the endangered Hawaiian tree snail *Achatinella*...


Figure 1. Sampling locations of snail feces and leaves. Samples were collected from four sites across the Waianae Mountain range on west Oahu, Hawaii.

Figure 2. Rank abundance of dominant OTUs. Figure 2a shows the 39 fungal OTUs and Figure 2b the 47 bacterial OTUs that contributed more than 0.3% of total sequencing reads.

Figure 3. NMDS plot illustrates how sample type and location structure (A) fungal and (B) bacterial community composition. Ellipses represent standard error of the mean (95%) for sampling locations illustrate the PERMANOVA findings that mean centroids do not overlap between the three key sites: Puu Hapapa, Palikea and (Pahole+Kahanahaiki). Pahole and Kahanahaiki which lie within 0.94 Km, are indistinguishable. Points are color-coded to indicate sample type, i.e. feces or leaf sample origin. Both fungi and bacteria samples were separated in ordination space by sample type, but location was the most important component of fungal community composition, rather than sample type or host plant identity. Stress values of the ordination are 0.21 (fungi), and 0.17 (bacteria).

Figure 4. PERMANOVA: Estimates of components of variation (\(\sqrt{V}\)). Location, sample type (feces or leaf) and host plant identity explained over 50% of the community composition variance. Host plant order had a similar affect on community composition for both bacteria and fungi (14.0% and 14.6%). Location was the strongest determinant of fungal composition (23.5% of
variance), whereas sample type and location contributed similarly to bacterial compositional structure (19.3% and 19.7%).

Figure 5. Gut dissection experiment demonstrates high overlap of microbial assemblages. Proportion of fungal DNA sequence reads detected in the gut, feces and phyllosphere associated with two Auriculella ambusta snails. Values in the overlapping regions refer to the percentage of reads that assign to OTUs detected in more than one sample. Most reads were detected in the union of the gut, feces and leaf samples.

Table 1. Samples collected from locations and host plants. Number and identity of samples collected from each location (rows), organized by host plant species (columns). For each sample, data were collected for fungal and bacterial communities present in snail feces and on leaf surfaces.

Table 2. PERMANOVA for bacterial and fungal diversity (Bray Curtis) across samples. A mixed effects model was used in which sample type and location were fixed and plant host was taken as a random factor. All levels significantly explained variance.

Table 3. OTUs significantly associated with either leaves (a) or feces (b).
Rows are filled in if an OTU is significantly associated with either feces or leaves. The “stat” column is the Indicspecies statistic that describes how good an indicator a particular OTU is for a particular combination of levels. “B” is the false
discovery rate, i.e., the likelihood that the OTU will occur in another level. The %
column refers to abundance of the OTU in the dataset, value following each
taxonomic assignment is a bootstrap statistic indicating the level of confidence in
that taxonomic assignment.
Figure 1

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