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

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Summary

Achatinella mustelina **is a critically endangered tree snail that subsists entirely by grazing microbes from leaf surfaces of native trees. Little is known about the 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 faecal 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 faeces and leaf samples, but the same microbes occurred in both. We conclude that 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 faecal samples reflect gut contents. Over 60% of fungal reads were shared between faeces, gut and leaf samples. Overall, location, sample type (faeces 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.**

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Introduction

The Hawaiian tree snail genus *Achatinella* once comprised approximately 41 species, all restricted to narrow ranges, endemic to the island of O'ahu. 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 (Holland *et al.*, 2012; Chiaverano and Holland, 2014), and habitat alteration and loss have severely impacted achatinelline species diversity and distributions. Today, only 10 species remain in the genus *Achatinella* (Holland and 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 O'ahu, and consists of six genetically distinct populations (Holland and 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 and 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 behaviour 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 faeces, which has been demonstrated with large herbivores and carnivorous predators (Parsons *et al*., 2005; Deagle *et al*., 2009; Barnett *et al*., 2010; Brown *et al*., 2011; Oehm *et al*., 2011).

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. Baker *et al*., 1979; Morris *et al*., 1998), and molecular characterization of the phyllosphere (e.g. Thompson *et al*., 1993; Knief *et al*., 2010). 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 and Jones, 2009; Vorholt, 2012; reviewed in Müller and Ruppel, 2013), 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 (Finkel *et al*., 2011; Kim *et al*., 2012; Kembel and Mueller, 2014).

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* faeces 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 faeces was correlated with that of the phyllosphere. To evaluate the extent to which microbes detected in the faeces 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 with that of their faeces and the phyllosphere. Finally, we discuss how novel molecular methods within a tritrophic ecological framework can abet basic natural history research for the conservation of an enigmatic endangered species.

Results

Sampling results

A total of 102 snail faeces/phyllosphere pairs were sampled from Pu'u 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 faecal/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 $18\,777 \pm 568$ per sample and for bacteria reads was 9435 ± 303. Four fungal samples and 11 bacterial samples had < 2000 reads and were removed from subsequent analysis. Total richness was high, with 7376 fungal OTUs and 3967 bacteria operational taxonomic units (OTUs) being detected after removal of singletons and rarefication 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 (Fig. 1A and B). 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 with 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 β-diversity

The community composition of microbes was structured by sample type (faeces/leaf), geographic locations from which samples were taken and taxonomic identity of the tree host (Table 2). Samples taken from the host plant *Myrsine 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 (Table S1A and B). 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

Table 1. Samples collected from locations and host plants.

Location	Date [dd-mm-yyyy]	Lat [°N]	Long [°W]	Elevation [m]	*Alyxia oliviformis*	*Antidesma sp.*	*Broussaisia arguta*	*Claoxylon sp.*	*Coprosma longifiora*	*Dianella sandwicensis*	*Diospyros sandwicensis*	*Hedyotis terminalis*	*Freycinetia arborea*	*Ilex anomala*	*Melicope oahuensis*	*Metrosideros polymorpha*	*Myrsine lessertiana*	*Myrsine sp.*	*Nestegis sandwicensis*	*Perrottetia sp.*	*Perrottetia sandwicensis*	*Pipturus albidus*	*Pisonia brunoniana*	*Pisonia umbellifera*	*Pisonia sandwicensis*	*Pittosporum glabra*	*Pouteria sandwicensis*	*Psychotria sp.*	*Schinus terebinthifolius*	*Smilax sp.*	*Psidium cattleianum*	*Urera glabra*	*Urera kaalae*
Pu'u Hapapa	07-12-2012	21°46.7	158°10.3	848				4					3	3			4	3		1		1	2	2				3	2	3	1		2	2
Palikea	15-01-2013	21°41.6	158°10.0	897		3	3		2	5		2	4		2	4	3				2	1						2	2		4		
Kahanahaiki	31-01-2013	21°53.8	158°19.5	671	3	1					1	2				1	4		3							1					3		
Pahole	31-01-2013	21°32.2	158°11.4	701					1										1						3								

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 faeces and on leaf surfaces.

remain significant factors, and geographic location remains the greatest factor that determines variance (Table S1C and D). 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 (Fig. 2). By contrast, location, host plant and sample type were largely equal components of variation for bacteria communities (Fig. 2).

Does faecal 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 faeces and not leaves (Table 3). However, there were no OTUs that occurred exclusively on either faeces or leaves. The β-diversity of fungal OTUs detected in faeces 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 faecal 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 faeces (Acidobacteriales, Enterobacteriales, Chthoniobacterales; Table 3B). Bacterial OTU_2 (Enterobacteriaceae) was the second most abundant bacterial OTU detected in the entire dataset (Fig. 1), has a likelihood of 93% of occurring in faeces, but only a 15% chance of occurring in the phyllosphere (Table 3).

Dissection experiment

Because snail faeces are nutrient-rich and moist, they likely provide an attractive substrate for environmental microbes. To determine the extent to which faecal microbial composition reflects gut composition, as opposed to exogenous colonization, two *Auriculella ambusta* were dissected and their gut contents compared with the leaf and faecal samples. Our dissection experiment showed that the majority of fungal sequencing reads (> 60%) were shared between the gut, leaf, and faeces, supporting the hypothesis that these phyllosphere microbes are passaged through the gut and detected in faeces (Fig. 3).

Discussion

What determines microbial composition of Achatinella *diet?*

Achatinella mustelina has the largest natural distribution (~ 24 km; Holland and Hadfield, 2002) within the genus,

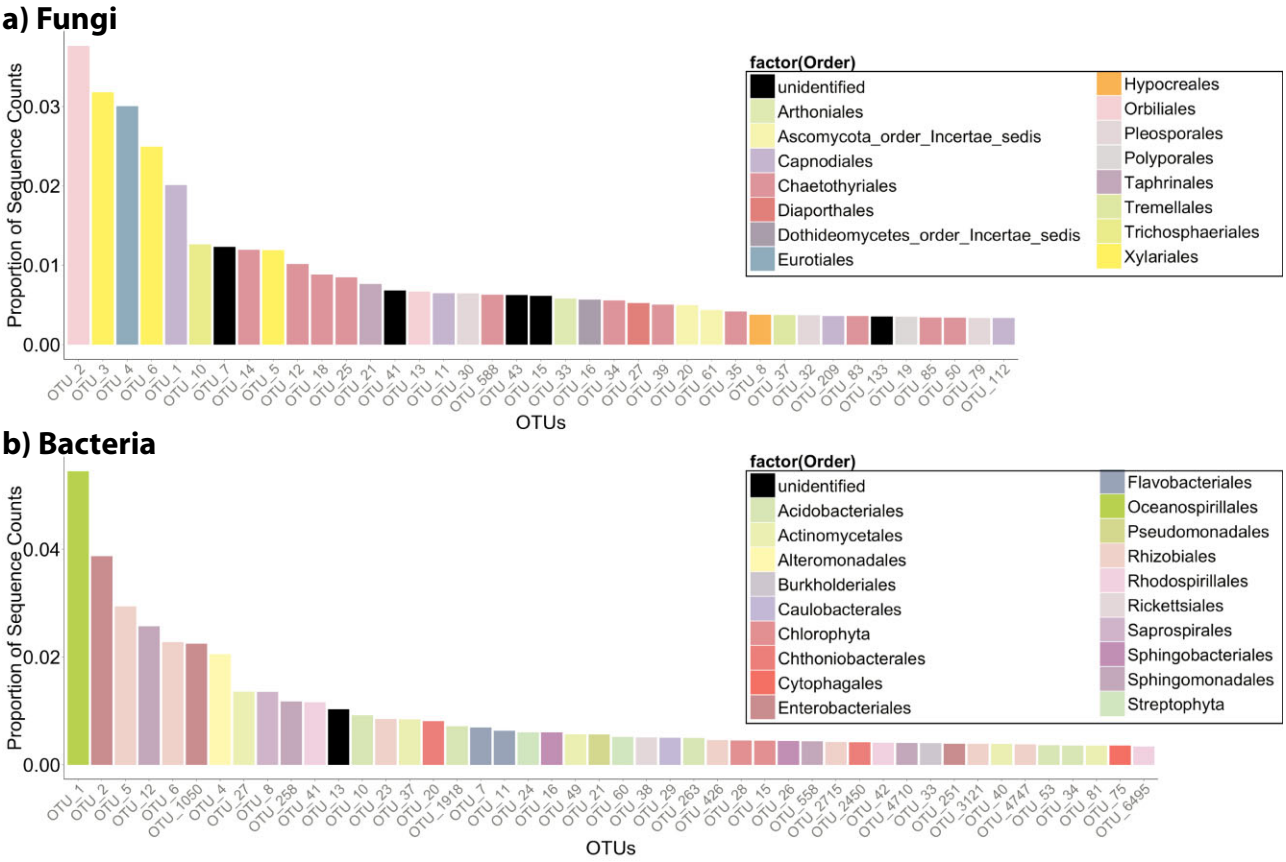

Fig. 1. Rank abundance of dominant OTUs. The figure shows (A) the 39 fungal OTUs and (B) the 47 bacterial OTUs that contributed more than 0.3 % of total sequencing reads.

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 (Marsh, 1966; Baker *et al.*, 1979), and culture-independent techniques (Zimmerman and 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

Table 2. PERMANOVA for bacterial and fungal diversity (Bray–Curtis) across samples.

Factors	Type III SS	df	MS	Pseudo-F	P(perm)
(A) Fungi					
Sample type[a]	2.812×10^4	1	2.812×10^4	10.188	1×10^{-4}
Location[a]	6.732×10^4	3	2.244×10^4	8.131	1×10^{-4}
Host plant order[b]	8.425×10^4	15	5.616×10^4	2.035	1×10^{-4}
Residual	4.747×10^5	172	2.760×10^3		
(B) Bacteria					
Sample type[a]	3.390×10^4	1	3.390×10^4	16.285	1×10^{-4}
Location[a]	3.614×10^4	3	1.205×10^4	5.7855	1×10^{-4}
Host plant order[b]	5.948×10^4	15	3.965×10^4	1.9044	1×10^{-4}
Residual	3.414×10^5	172	2.082×10^3		

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.
a. Fixed factor.
b. Random factor.

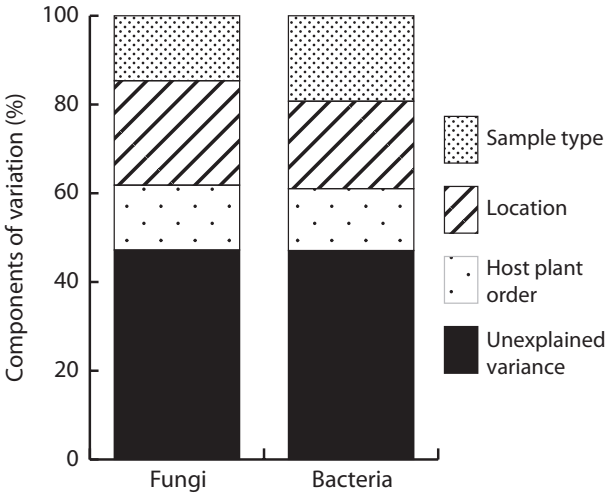

Fig. 2. PERMANOVA: Estimates of components of variation (√V). Location, sample type (faeces or leaf) and host plant identity explained over 50% of the community composition variance. Host plant order had a similar affect on community composition for both bacteria and fungi (14.0% and 14.6%). Location 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%).

species (Marsh, 1966; Baker *et al*., 1979; Zimmerman and 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 and 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 faecal composition correlate with phyllosphere composition?

One objective of the present study was to assess the extent to which the microbial composition of snail faeces 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 faeces, and only a few OTUs were indicators for faeces or phyllosphere communities (Table 3). Furthermore, the dissection experiment of *Auriculella ambusta* confirmed that OTUs detected in the faeces 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 faeces or leaves, and that there were few microbes that were more likely to be associated with either faeces or leaves (Table 3). There is also considerable overlap in microbe identity found in gut contents, faeces, and leaves (Fig. 3). However, the community frequency distributions also appear to be skewed by the digestive process (Fig. 4 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*., 2010; 2013). 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 faeces 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 Hawaii Island, which varied from 223 to 258 OTUs per rarefied sample (Zimmerman and Vitousek, 2012), and comparable to the Barro Colorado phyllosphere study: 279 ± 6 (excluding OTUs with < 10 reads; Kembel and Mueller, 2014). The OTU distributions of both bacteria and fungi conformed to log-normal distributions (Fig. 1A and B), 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).

Table 3. OTUs significantly associated with either leaves (A) or faeces (B).

OTU	Faecal	Leaf	B	Stat	%	Class		Order		Family		Genus	
(A)													
Ascomycetes													
OTU_14	1	0	0.87	0.86***	**1.19**	Eurotiomycetes	90	Chaetothyriales	87		NA		NA
OTU_588	1	0	0.80	0.83***	0.63	Eurotiomycetes	80	Chaetothyriales	79		NA		NA
(B)													
Acidobacteria													
OTU_101	1	0	0.80	0.81***	0.32	Acidobacteriia	100	Acidobacteriales	100	Acidobacteriaceae	100	Edaphobacter	76
OTU_68	0	1	0.67	0.81***	0.18	Actinobacteria	100	Actinomycetales	100	Geodermatophilaceae	93	Geodermatophilus	80
OTU_81	0	1	0.93	0.91***	0.36	Actinobacteria	100	Actinomycetales	100	Microbacteriaceae	94	Yonghaparkia	71
OTU_1766	0	1	0.70	0.82***	0.14	Actinobacteria	100	Actinomycetales	100				
Bacteroidetes													
OTU_75	0	1	0.81	0.88***	0.36	Cytophagia	100	Cytophagales	100	Cytophagaceae	100	Hymenobacter	100
OTU_418	0	1	0.80	0.86***	0.17	Cytophagia	100	Cytophagales	100	Cytophagaceae	100	Hymenobacter	100
OTU_170	0	1	0.71	0.82***	0.12	Cytophagia	100	Cytophagales	100	Cytophagaceae	100	Hymenobacter	100
OTU_249	0	1	0.74	0.84***	0.11	Cytophagia	99	Cytophagales	99	Cytophagaceae	99	Hymenobacter	99
OTU_113	0	1	0.72	0.83***	0.23	Cytophagia	100	Cytophagales	100	Cytophagaceae	100	Spirosoma	100
OTU_90	0	1	0.72	0.83***	0.32	Saprospirae	100	Saprospirales	100	Chitinophagaceae	100		
Proteobacteria													
OTU_2	1	0	0.85	0.89***	**3.88**	Gammaproteobacteria	100	Enterobacteriales	100	Enterobacteriaceae	100		
Verrucomicrobia													
OTU_145	1	0	0.86	0.84***	0.31	Spartobacteria	100	Chthoniobacterales	100	Chthoniobacteraceae	95	Candidatus	91

Rows are filled in if an OTU is significantly associated with either faeces 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 and asterisks (***) indicate a high level of significance (≤ 0.001). '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.

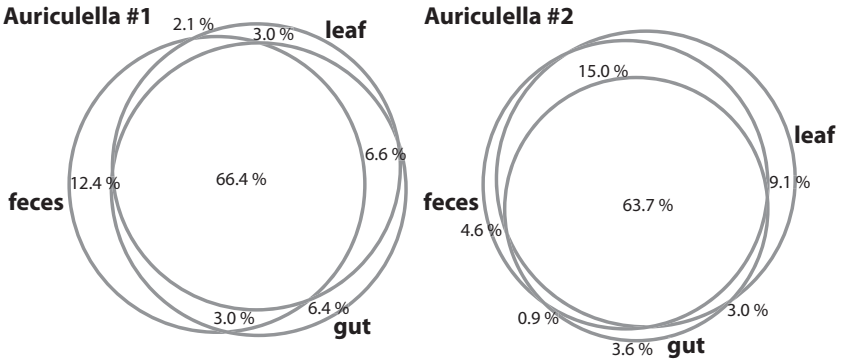

Fig. 3. Gut dissection experiment demonstrates high overlap of microbial assemblages. Proportion of fungal DNA sequence reads detected in the gut, faeces and phyllosphere associated with two *Auriculella ambusta* snails. Values in the overlapping regions refer to the percentage of reads that assign to OTUs detected in more than one sample. Most reads were detected in the union of the gut, faeces and leaf samples.

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 and 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 (Baker *et al*., 1979; Kembel and Mueller, 2014), 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, Enterobacteriaes; and OTU_4, Alteromonadales), which comprised 17% of reads. Of the Gammaproteobacteria, the Enterobacteriales (e.g. OTU_2) were most abundant in faecal 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 and 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 (Delmotte *et al*., 2009; Redford *et al*., 2010); some can fix nitrogen (e.g. *Beijerinckia*) and many can metabolize C_1 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 faecal samples. For example, OTUs 12 and 258 from the diverse order

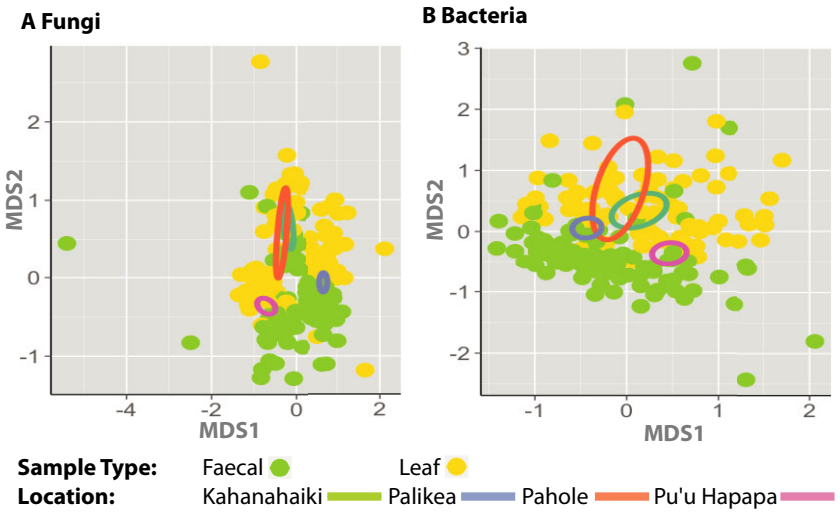

Fig. 4. NMDS plot illustrates how sample type and location structure (A) fungal and (B) bacterial community composition. Ellipses represent standard error of the mean (95%) for sampling locations and illustrate the PERMANOVA findings that mean centroids do not overlap between the three key Points are colour-coded to indicate sample type, i.e. faeces 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).

Sphingomonadales, known for the ability to metabolize complex polymers, were also ubiquitous (Delmotte *et al*., 2009; Redford *et al*., 2010). Therefore, although the present study does not corroborate Baker and colleagues (1979) in their inference that 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 (Rollins *et al*., 2001; Poulsen and Boomsma, 2005; Silliman and Newell, 2011; Aizenberg-Gershtein *et al*., 2013; 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 (Lindow and Brandl, 2003; Yadav *et al*., 2005). Mucus secreted during terrestrial gastropod locomotion, know as the slime trail, has been shown to have selective antimicrobial properties (Iguchi *et al*., 1982; 1985; Kubota *et al*., 1985). A third potentially important factor in determining arboreal microbial community structure could be that tree snail faeces recycle, deposit and fertilize fungal spores back into the phyllosphere. This process may play an as yet undocumented role in the determination and maintenance of microbial community structure. The fungal phyllosphere is presumably horizontally inherited (Osono and Mori, 2003), 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 faeces 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 and Hadfield, 1996). The effects 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 faecal 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.

Experimental procedures

Sampling

Sampling stations and dates are given in Table 1. To ensure the sampled faeces were recently deposited, tree foliage was searched at nighttime for snails, and the trees in which snails were present were flagged. *Achatinella* faeces 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 faeces on a range of host plant species at each site. Snail faeces 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 Pu'u Hapapa station (Fig. 5) and transported along with host leaves in a container so that the gut could be dissected under laboratory conditions.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Genomic DNA from faeces, leaf swabs and the *Auriculella ambusta* gut were extracted using a commercially available

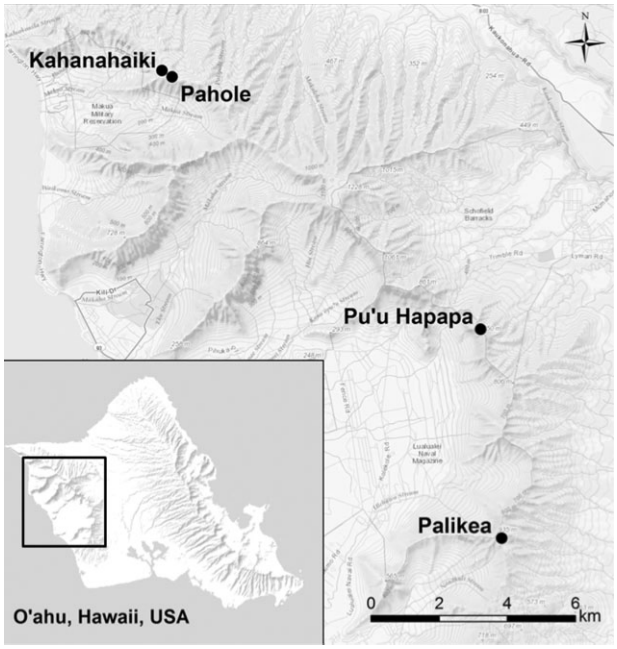

Fig. 5. Sampling locations of snail faeces and leaves. Samples were collected from four sites across the Waianae Mountain range on west O'ahu, Hawaii.

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.

Polymerase chain 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 programmes 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). Polymerase chain reactions 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). Polymerase chain reaction 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 an SPRI plate (Beckman Coulter, California) and Sera-Mag Speedbeads (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 3:2 ratio (as per the recommendation of Smith and 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 300 bp 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 sequence read archive (SRA) under accessions SAMN03019997 – SAMN03020200.

Sequence processing

Full processing pipeline commands are available online as supplementary materials (S2), 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 .taxonomy files in Supplemental Scripts in S2).

Data analyses

Apart from the mixed-effects model analyses, which were conducted in PRIMER-6 (Clarke and Warwick, 2005), data were analysed in R using the packages VEGAN (Dixon, 2009), MASS (Venables and Ripley, 2002) and INDICSPECIES (De Cáceres and Legendre, 2009), and were visualized with GGPLOT2 (Wickham, 2009). Full scripts are available as supplementary materials (.R file in S2). Libraries were rarefied to 2000 reads and samples with less than 2000 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 and Curtis, 1957). Correlation between faecal and leaf matrices were assessed using a Mantel test (Mantel, 1967). The influence of different factors on variance were then visualized by non-metric multidimensional scaling NMDS ordinations and modelled 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 and Legendre, 2009).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. 1a and 1b analyze variance of community samples that were taken from a plant, *Myrsine lessertania*, which occurs across the three major sampling sites. 1c and 1d model sample variance using only those three host-plant orders that are present across the three major sites.