Molecular assessment of wild *Achatinella mustelina* diet

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*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 believe that diet in captivity may be a limiting factor. 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 frass, since digested microbes are difficult to identify visually.

The proposed project is to assess the use of molecular methods to investigate:

1) The diet of wild *Achatinella mustelina* in Oahu’s Waianae mountain range
2) The extent to which microbial species present in snail frass correlate with microbial species present on leaf surfaces where snails are encountered
3) Whether microbial composition of frass and/or leaf surface corresponds with location or plant host identity.

**Samples**

One hundred forty two field samples of *A. mustelina* frass, 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 Kahanahaiki 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 snail) were also collected from Makaha and dissected in order to compare gut contents with leaf surface and frass microbial diversity.
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 frass and leaves were extracted using a commercially available kit (MO-BIO PowerSoil). Frass was added to maceration tubes directly, whereas microbial biofilms from the leaf surface were sampled using sterile swabs. Both frass and leaf samples were PCR-amplified using DNA primers specific for Fungal (ITS1f and ITS4) and Bacterial (515f and 907r) barcode regions. Each sample is PCR-amplified using a short, unique “tag” sequence of nucleotides in order that sample identity is maintained throughout the analysis. These primers will 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/frass rather than lab contamination.

### Number and identity of samples collected from each location (rows), organized by host plant (columns). For each sample data was collected on fungal and bacterial communities present in snail frass and on leaf surfaces.

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Sequencing and “Barcode Analysis” of Microbial Communities

PCR products from Bacteria and Fungal reactions are in cue to be sequenced on an Illumina MiSEQ platform. The results from this technology are analogous to data derived from cloning and sequencing in that single-stranded reads are generated from a mixed template, however a single 10-hour run generates approximately 20,000,000 sequences. This increased throughput is useful for assessing the composition of complex microbial communities present in natural systems.

Computationaly expensive bioinformatic analyses will make use of the QIIME analytical platform as implemented in Amazon’s EC2 cloud computing environment. Sequenced microbes will be identified by comparing their DNA sequences to identified strains deposited in sequence databases such as NCBI GenBank. We hypothesize that microbes present in both phyllosphere and frass samples are components of A. mustelina diets. Our analysis will also enable a rapid assessment of which microbes, if any, are universally associated with wild A. mustelina frass, as these may suggest important diet supplements for captive rearing programs.

Dr. Richard O’Rorke, a post-doctoral researcher has been hired to complete the bio-informatic analyses.
Example PCR gel. Negative control (swab) at far left. DNA ladder at right. PCR products are ~350 bp. Smears at the bottom of the gel are primer dimer and are removed prior to sequencing.

Potential Pitfalls and Likelihood of a Successful outcome

Methods employed in this project are proven, so there is little doubt that we will obtain the data required. The greatest risk is that our results will be equivocal. There is the potential that there will be no overlap between phyllosphere and frass communities, so that is unclear whether detected microbes were actually consumed by the animal or arrived after deposition on the leaf surface. In this even we can alter our sampling to ensure that only fresh frass is collected, or else consider alternative methodologies (such as manipulative transplants in the lab) to assess more directly how host plant and phyllosphere affect diet.

A secondary risk is that little of the multivariate microbial community data variance will be partitioned by measured variables (source, location host plant). Such risks exist in any pilot study, and even these negative results can be informative.

Next Steps and Future Directions

1) Analyze and synthesize data generated during year 1 in order to identify likely food sources for *A. mustelina*, and the extent to which those food sources differ according to geographic location and host plant.
2) **Cultivate** a broad assemblage of epiphytic microbes to supplement the single strain currently available for captive endangered tree snail diet.

3) **Refine sampling** effort and data collection as warranted given the results of Objective 1. If necessary, collect additional data.

4) **Synthesize and present** results to OANRP, snail conservationists and others. Given the probable high lineage diversity, and likelihood of endemic/undescribed microbes in tree snail diets, results from this study may be considered "parochial". We plan, therefor, a polished and thoughtful synthesis for conservationists and managers.