APPENDIX ES-4

Molecular assessment of wild *Achatinella mustelina* diet

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Food Similarity Between Proposed Donor and Enclosure Snail Sites

If populations of *Achatinella mustelina* in difficult-to-access areas are to be successfully relocated to enclosures at sites more amenable to conservation efforts, it must be assured that conditions at the proposed sites are similar to those where the snails currently reside. One factor that may be important is the availability of preferred snail food sources. We are determining whether epiphytic microbial communities are similar between donor and proposed enclosure sites by sequencing DNA amplicons of material swabbed from the surface of leaves at each location. At each current and proposed snail site, leaves from at least 10 plants were recorded, collected and returned to the lab. In the lab, leaf surfaces were swabbed and these swabs were subjected to DNA sequencing to determine species composition. If leaf-surface microbial communities are similar between current and proposed sites, it is an indication that food source and availability will not be limiting factors in snail health at proposed sites following translocation. If microbial communities are dissimilar, further work will be done to determine whether these differences are functionally meaningful and/or whether it is possible to inoculate plant surfaces at the proposed sites with microbial food sources from the current sites to ease any potential snail relocation shock.

Donor Site Proposed Site
Skeet Pass - Ka'ala Bog
Culvert 69 - Three Points
Ekahanui - Palikea Area

Fungal ITS genetic marker regions were amplified and sequenced at the University of Hawaii HIMB Genetics Core Facility. The resulting reads were then combined into probable operational taxonomic units which were used to construct dissimilarity measures between sites.

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Figure 1 - Map of sampling locations (Waianae range)

Results

There was a significant difference (Anosim; P=0.001) in epiphyte fungal community structure between the four sites near Kaala summit (Kaala Bog, Culvert 69, Skeet Pass, and Three-Points) and the three sites near Palikea (DS Palikea, Kaaikukai, and Ekahanui). There was no detectable difference between any of the current/proposed site pairings, however (See Fig. 3). Thus, it does not seem likely that any snails moved to proposed enclosure locations will encounter significantly different food sources from their currently paired extant sites and food sources will not be limiting factors in snail health following translocation.

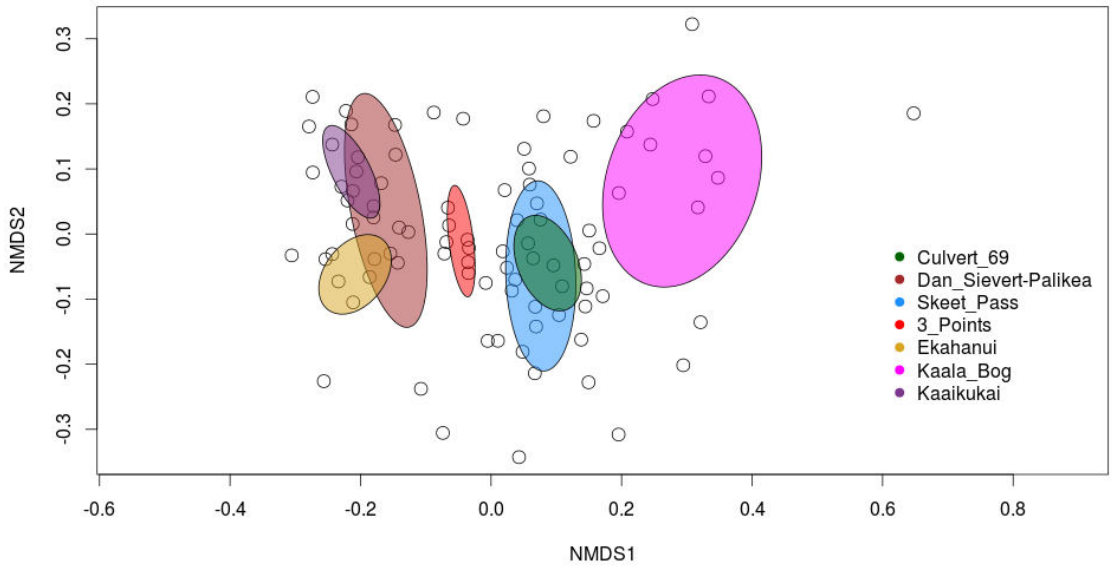
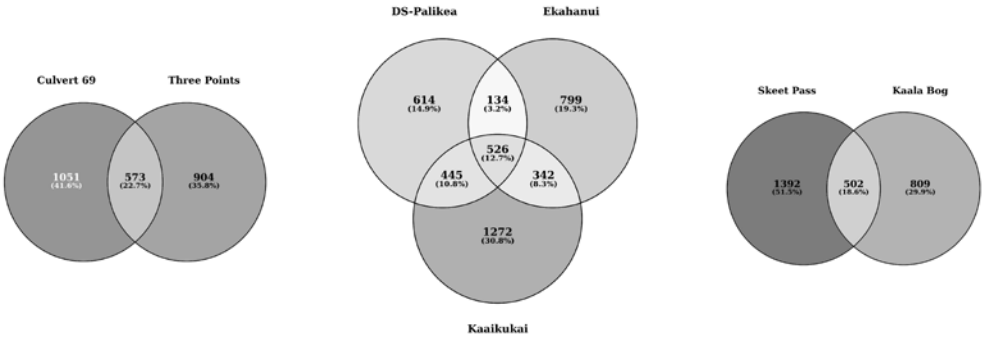


Figure 2 - NMDS projection of epiphyte fungal communities. Ellipses represent the standard deviation of point scores around the group centroid.

Phyllostegia endophytes and pathogen resistance

Phyllostegia mollis and *Phyllostegia kaalaensis* are federally listed endangered plant species endemic to Oahu, HI. There are currently no known wild populations of *P. kaalaensis* and the few wild populations of *P. mollis* are failing to demonstrate long-term survival. Greenhouse populations of these plants are maintained by the Army Natural Resources division, but they show marked susceptibility to fungal pathogens, particularly the powdery mildew, *Neoerysiphe galeopsidis*. Greenhouse populations are, therefore, dependent on regular fungicide treatments which are impossible to maintain once individuals have been out-planted to habitats within their native ranges. Current scientific consensus is that the fungi which coexist within plant tissues form an integral part of plant fitness. These beneficial endophytic and mycorrhizal fungi are not present in plants that have received regular fungicidal treatments, so they are not present in out-planted populations of *P. mollis* or *P. kaalaensis*. One of the major benefits that host plants receive from mutualistic fungi is increased resistance to disease, as mutualistic fungi can outcompete pathogens for habitable living space or even actively repel invasive fungi through excreting chemical compounds. The essentially sterile plants are presumed, therefore, to be highly susceptible to attack by pathogenic fungi in the environment. We have completed a pilot study on the efficacy of transplanting fungal endophytes from healthy wild populations of *P. mollis* and *P. hirsuta* into



ANOSIM statistic R: -0.03114
Significance: 0.619

ANOSIM statistic R: -0.06877
Significance: 0.837

ANOSIM statistic R: -0.08435
Significance: 0.91

Figure 3 Shared OTUs between current/proposed site pairings. The proportions of OTUs unique to each site do not constitute statistically significant differences

greenhouse-raised *P. mollis* and *P. kaalaensis* individuals. Two experimental transplantation methods were tested: 1) Isolating individual fungal strains from wild hosts, culturing them in the lab, and spraying them onto the leaves of greenhouse-raised individuals; 2) Preparing a low-tech slurry from leaves of wild individuals, filtering out large particles, and spraying this onto the new host plants. The first method has the benefit that we know exactly what we are applying to the new host leaves, the second method has the benefit of potentially passing on beneficial fungi that are not amenable to laboratory culture. Preliminary results were intriguing. The cultured fungal isolates did not appear to confer any advantage over the control group with respect to disease severity, but the group receiving the slurry of wild leaves showed delayed mortality and decreased disease severity for a time (Fig. 4). By the end of one month all plants had generally succumbed to *N. galeopsidis* but the "leaf slurry" treatment warrants further investigation, as it showed some benefit, at least for the first three weeks. By this time the pathogen load on the other two treatments was essentially 100%, with all leaf surfaces covered with sporulating fungus and the slurry-treated plants, in such close proximity, did not last long after. We are nearing the end of a second round of tests, and the results are similar and even more pronounced. DNA from the inoculae and the initial plant endophyte loads was sequenced and the results are surprising. Roughly 90% of the fungal reads from the leaf slurry treatment, which is showing so much promise, come from *N. galeopsidis,* the same pathogen that appears to be killing the plants (Fig. 5). Leaf samples have been taken at regular intervals during both rounds of testing to track the colonization of plant tissues by fungal inoculae. When these samples are sequenced it will be clearer what fungi were able to establish in the plants, and whether the plants treated with the leaf slurry have been colonized by any strains of *N. galeopsidis*.

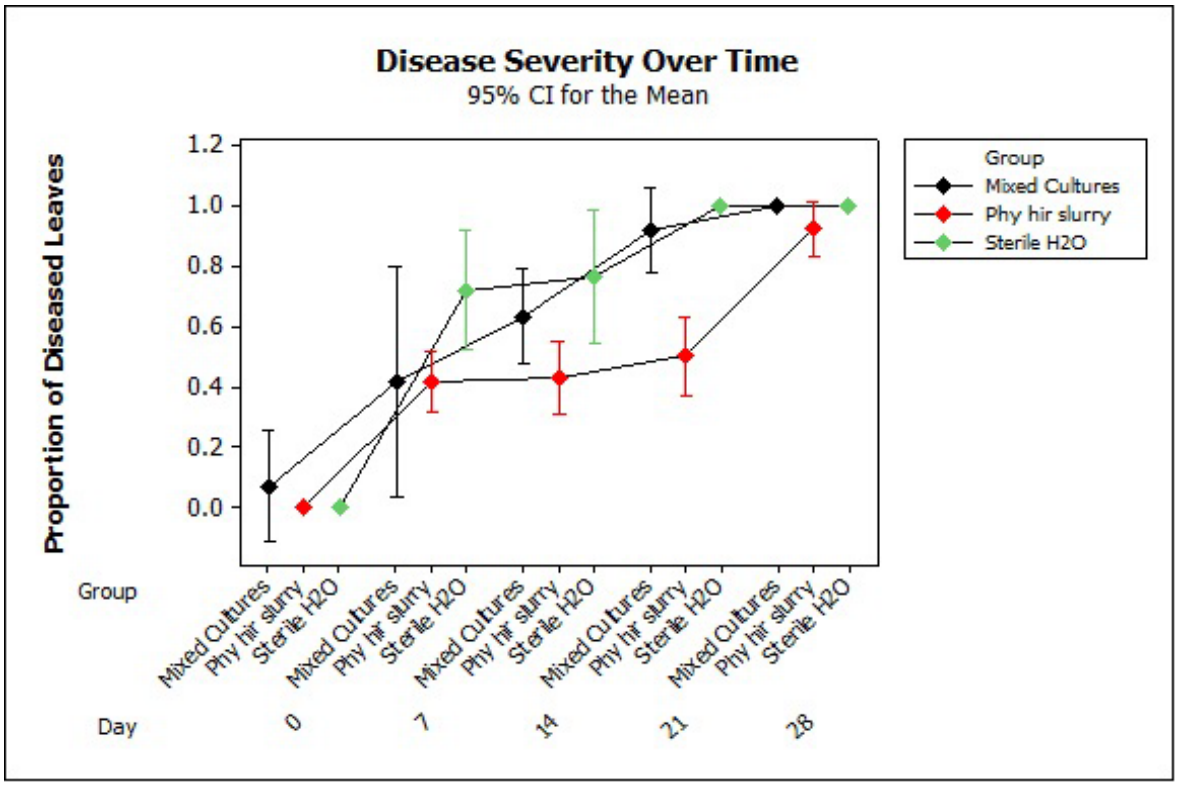


Figure 4 - The proportion of diseased leaves over time. The slurry from healthy wild leaves (shown in red) conferred a longer time until full onset of disease.

Other work

Captive (laboratory) snail (*Achatinella mustelina)* populations are dependent on microbes from wild leaves to supplement their diet. These leaves are obtained by regular field forays which are costly and time consuming. The ability to grow diverse microbial communities on laboratory-amenable plants would be a major convenience for maintaining healthy laboratory snail populations. We are in the early stages of investigating the efficacy of such a system using the model plant *Arabidopsis thaliana*. An initial study is under way to examine the factors that determine the composition of a newly-forming microbial community, such as would be seeded onto the plants in order to grow "snail food." This plant is very fast growing and there are thousands of curated ecotypes that display a wide range of phenotypic traits, so it potentially offers a highly customizable "delivery system" for supplementing snail captive diets without constant trips into the field.

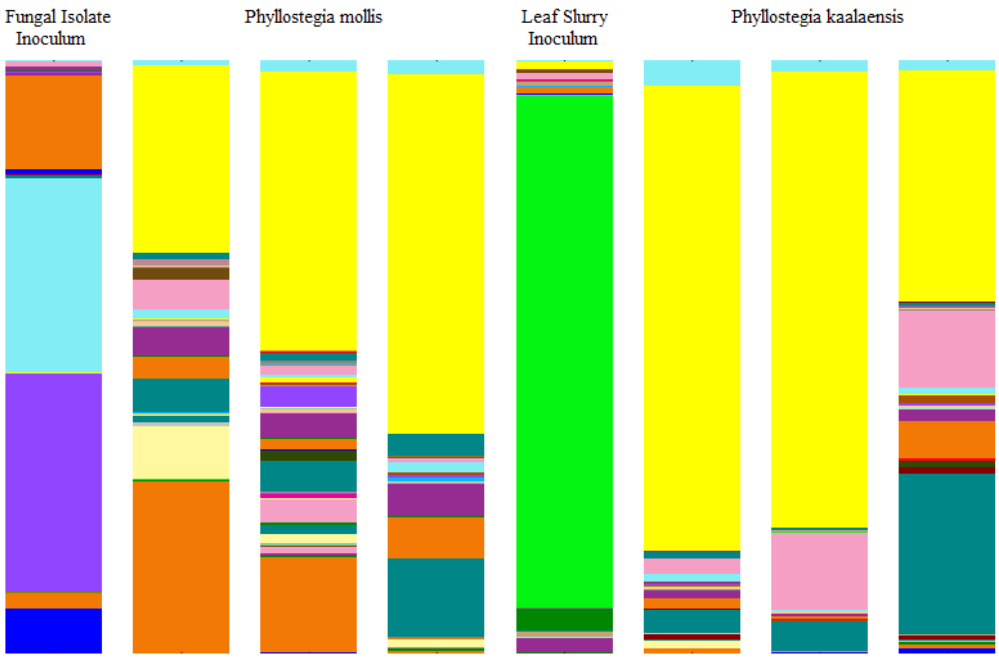


Figure 5 - Stacked bar chart of fungal species identities in initial starting conditions of Phyllostegia experiment. The two inoculae, and three replicates of each plant species. The bright green bar in the Leaf Slurry Inoculum represents N. galeopsidis.

APPENDIX ES-5

ADAPTIVE GENETICS OF HAWAIIAN TREE SNAILS & CLIMATE CHANGE

Final Quarter Report, 2015

Dr. Michael Hadfield & Dr. Melissa Price

Accomplishments

Whole mitochondrial genomes have been compared across the range of *Achatinella mustelina*. These results suggest the same management approach as COI alone (Holland and Hadfield's 2002 paper), suggesting no change to the current management approach of 5 or 6 discrete ESUs, with populations grouping along the Waianae ridgelines.

However, when nuclear evidence was considered (a scan/survey of thousands of sites across the entire genome), we observed a more nuanced picture. For example, Makaha (ESU D) always groups with Koiahi and Ohikilolo (ESU B). Puu Hapapa (ESU D) groups with Ekahanui (ESU E) about 50% of the time. On the other hand, some populations are very much the same for both nuclear and mitochondrial markers. ESU C (Haleauau and Skeet Pass) always groups together, separate from the others. The populations on the three ridges that meet on top of Mt. Kaala (from ESUs B, C, D) separate out from one another with both mitochondrial and whole-genome approaches.

Based on these initial results, populations that are far apart geographically, even though they are lumped in the same ESU based on mitochondrial gene sequences, should NOT be lumped into the same enclosure simply because they are in the same ESU, particularly for ESUs B and D, which stretch a considerable distance. Unsurprisingly, total DNA evidence suggests that snail populations that are closer together geographically are more closely related genetically, and things that are farther apart are less related. Pulling nearby populations into enclosures should be enough to combat inbreeding, if that is the goal. In light of climate change, we still recommend ONLY moving snails to wetter, cooler locations, and never to locations that are warmer or drier than source locations.

GIS modeling has been scaled down to the level of ESUs. The climate-change modeling results, which have now been projected for both 25 and 60 years, suggest urgent management actions will be necessary in the near future, but we are not ready to make a specific recommendation. We may need to start intentionally mixing populations to help with adaptation to climate change. If populations are mixed for this purpose, individuals must ALWAYS be moved from drier, warmer environments to wetter, cooler environments, and not the other way around.

Forecast

Continued work with SNP identification and Fst-outlier analysis will be used to identify SNPs correlated with environmental variables. These data will be combined with the species' current-range data, as well as forecast data, to predict where populations will be likely to tolerate warmer, drier conditions, and which populations should be combined to maximize adaptive ability.