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## Intro:

The intensity and types of environmental challenges facing plants vary at all spatial scales, from landscape-level scales down to variability among different regions of individual plant bodies. Temporal changes in environmental challenges to plants also range from hourly, to seasonal or even decadal fluctuations in climate, pathogen loads, nutrient availability, and exposure. Plant genomes contain many solutions to these challenges, especially to environmental challenges which have acted as long-term selective pressures on plants. In these times of rapid change, however, the abundance and rhythm of all types of plant stressors has increased, and plant genomes alone may not evolve quickly enough to ensure the success of ecologically or socially important plants in the face of increased pathogen loads, decreased climatic stability, and decreased habitat availability.

Some have proposed that the plant microbiome, with its collective abundance of microbial genetic resources, may represent an important and adaptive addition to the host plant genome. Together, it is proposed, the microbiome and the plant sum to make up the plant “hologenome”, which is more extensive and more dynamic than the plant genome alone. It remains a mystery how historically important hologenomes were in shaping plant populations, even prior to the current era of rapid anthropogenic change. However, evidence has mounted rapidly that microbiomes are important determinants in plant distributions and ecosystem health. If microbiomes are to be harnessed in any way to help plant health in times of rapid change, their spatial and temporal patterns must be better understood.

## Pertinence to research on-going at Smithsonian Institute (SI) and Smithsonian Tropical Research Institute (STRI):

One of the greatest contributions to tropical ecology in recent memory by SI and STRI is the empirical testing of the Janzen-Connell hypothesis (JCH), and exploration of the mechanisms behind the negative density dependence of seedling success as predicted by the JCH. This work will add to this ongoing effort to exploring this natural pattern in tropical forests. Mangan et al.<sup>1</sup> found that soil-born enemies may be main culprits in the negative density dependence patterns of rare trees in the tropics, but noted that highly abundant trees do not seem to display the same vulnerabilities to pathogens from congeners that rare trees do. The mechanisms at work in abundant tree species in overcoming the limiting of the JCH are still largely unexplained, though one group has found that small population sizes of rare trees may be subject to inadequate R gene diversity and gene flow<sup>2</sup> to keep up in the pathogen arms race. Here we suggest another, complementary mechanism - if functional microbiomes are key to pathogen resistance (as suggested here by us and elsewhere by numerous others), **then recruitment of beneficial microorganisms into the microbiome may represent a positive-density-dependent feedback.** Abundant organisms may be able to transmit their microbiomes to seedlings more successfully<sup>3</sup>, causing positive-density-dependent feedbacks in seedling success, and clusters of abundant species may represent larger “target” areas not only for pathogens but for environmental recruitment of beneficial microbial partners. As just one example, Rock-Blake et al.<sup>4</sup> found hypersensitivity by host plants to availability of symbiotic soil fungal partners on the landscape. This indicates that the availability of mutualistic symbioses may be important to overcoming the inhibitory effects of soil-born enemies and the negative density: both plant and fungal mycorrhizal partners

presumably benefit by the presence of the other. This would therefore represent a possible positive-density-dependent feedback that could counter the effects of soil-born fungal pathogens.

## Methods:

Here we propose to undertake sampling for the functional microbiome of a host tree species, *Eugenia nesiotica*, across the extent of the BCI ForestGEO plot. Endophytes from both leaf and root tissue of *Eugenia* will be surveyed for the presence of biosynthetic gene clusters, using two survey methods:

### PKS and NRPS metabarcoding

A metabarcode study will broadly target polyketide synthases (PKS) and non-ribosomal peptide synthases (NRPS) with degenerate primers. These data will be engaged to get a general picture of some broad classes of secondary metabolites that tree microbiomes are predicted to be producing differentially in response to environmental challenges. These enzymes encode backbone molecules for two of the broadest families of microbial secondary metabolites (polyketides and non-ribosomal peptides)<sup>5,6</sup>, and exist in diverse homologs that can be used to infer medium-to-fine grain portrait of the secondary metabolome of the plant microbiome. Amplicon library preparation will follow Thomas et al.<sup>7</sup>, for sequencing on Illumina MiSeq platform. Primer pairs for this survey will be based on Charlop-Powers et al.<sup>8</sup>. Preliminary surveys of these primers on public databases show very few matches to plant sequences (see jupyter notebook). Though most observations of NRPS and type I PKSes originate from microbial sequences, the possibility exists that that plants are greatly undersampled for type I PKS gene clusters<sup>9</sup>. Since our hypothesis focuses on the complementary role of the microbiome, we will attempt enhance the PKS- and NRPS-targeting primers with plant-blocking oligonucleotides<sup>10</sup>.

### Direct metabolite PCR tests

Some key metabolites may not be detected in our metabarcode survey, either because they are synthesized using a peptide or polyketide backbone, or because of sampling error. Our metabarcode survey will be augmented with more targeted PCR surveys for the presence of biosynthetic pathways of select microbial products. Since we hypothesize that host plants may selectively recruit microbial symbionts that produce secondary metabolites that enhance tolerance or resistance to environmental stressors and disease, we will develop primer sets for detection of key metabolic steps in the synthesis of:

1. Ergot alkaloids and rugulosins (anti-herbivory response)
2. Microbial antioxidants superoxide dismutase and catalase (drought stress response)
3. Fungal anti-mycotic compounds chinocandins, polyenols, and strobilurins (pathogen response)

Existing literature and public databases will be used to develop pathway-specific primers each of these enzyme synthesis pathways.

### Sampling scheme:

In both survey methods, spatial analysis of the presence of “useful” metabolites produced by plant-associated microbes will be coupled with microsite data, estimates of disease and herbivory load, and

the proximity of conspecific trees. Leaf and root tissue will be collected at 36 locations within the plot according to a spatial grid with a grain of 100m. Epiphyte and endophyte microbial populations will be sampled. Aerial plant tissues will be sampled at upper and lower canopy sites, to test microsite variability in microbiome expression due to position in the canopy.

### **Sample preparation:**

Roots will be gently brushed clean of soil using a brush. To sample epiphytes of leaves and roots, leaves and root sections will be submerged in 20 mL of 0.1% tween / sterile water mixture, and vortexed for 5 minutes. This wash fluid will be pelletized and frozen for later DNA extraction. Root and leaf endophytes sampling will follow Thomas et al.<sup>7</sup>, using a bleach-based surface sterilization method, followed by tissue homogenization and DNA extraction. Bulk DNA will be purified from host plant tissue and epiphyte wash-pellets using column kits, and selectively PCR amplified with above primers.

Plant pathogen loads and damage from herbivory will be phenotyped from photos<sup>11</sup> of 10 randomly selected from leaves from upper and lower zones of the tree, in addition to photos of leaves harvested for endophyte and epiphyte sampling. 50 grams of soil from each tree will also be harvested for soil moisture estimates.

### **Bioinformatic and Statistical Analyses:**

Metabarcoding results will be subject to standard bioinformatic pipelines to reduce sequencing artefacts and group highly similar reads<sup>12</sup>. Following this, amplicon reads were assigned to known biosynthetic gene clusters using the software package eSNaPD<sup>8,13</sup> and public databases. The resulting gene-cluster-by-sample-site matrix will be examined standard numerical ecology-based methods for modeling large matrices as dependent objects of environmental data (“regression” of metabarcoding results against environmental data and spatial patterns using redundancy analyses, including MEM and variation-partitioning methods)<sup>14–17</sup>. Read libraries will also be mined for specific biosynthetic pathways of interest (alkaloids, antibiotics, antioxidants, osmolytes, etc), to the level of specificity allowed by the homology of the polyketide and peptide backbones. and these will be treated as point patterns<sup>18</sup>, along with the results of the targeted PCR tests. Point patterns of secondary products of interest will be analyzed for cooccurrence with environmental challenges, herbivory, pathogen loads, and proximity and size of conspecifics and cluster size of nearest conspecifics.

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