

Metagenomics of Glen Rose Soil Microbial Systems and Their Roles in Carbon  
Cycling and Native Plant Restoration

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## Abstract

Central Texas has a region of uplifted limestone, the Edwards Plateau, providing an island of glen rose soils that fostered the speciation of many organisms and forms one of North America's areas of endemism. With an extreme and unpredictably variable climate, and alkaline soils, the survival of regional flora is increasingly recognized to be dependent on tight relationships with soil microbial populations, none of which have been described. The soil biota project proposed will fall within the framework of the development of multiple species monitoring approaches for HCPs and we will follow standards now used in other HCPs (Barrows et al. 2005).

Over the summer of 2015 root microbiome was sampled from seven plant species: *Muhlenbergia reverchonii*, *Yucca rupicola*, *Sophora secundiflora*, *Juniperus ashei*, *Arbutus xalapensis*, *Nolina lindheimeri*, *Prosopis glandulosa* from the Wild Basin Creative Research Center. Microbial (bacterial, fungal) DNA samples derived from four samples associated with each species are currently being sequenced. In addition, a preliminary analysis of the microbial DNA sequences obtained from four species sampled Summer 2014 (*Muhlenbergia reverchonii*, *Carex planostachys*, *Schizachyrium scoparium*, *Juniperus ashei*) reveals potential recruitment of specific bacterial populations to rhizosphere and endosphere for each species.

Characterization of rhizosphere and endosphere microbial communities associated with endemic plants typical of black-capped vireo habitat will potentially inform restoration efforts regarding the requirements for these communities in efforts to restore/expand these habitats.

## Hypotheses:

1. Soil microbial systems play key roles in the establishment, maintenance and viability of plant communities.
2. Rhizosphere (plant-root interface) microbial diversity is influenced by physical and chemical properties of the rhizosphere, some of which may be determined by the host plant.

## Objectives:

1. Characterize microbial communities (Fungal, eubacterial) characteristic of Glen Rose Soils (bulk) present within BPC.
2. Characterize microbial communities (Fungal, eubacterial) present within rhizosphere of plants that define Black-capped vireo habitat.

## Introduction

Soil microorganisms constitute a significant fraction of the Earth's biomass, with surface soils estimated to contain  $10^9$ - $10^{10}$  microbial cells per gram including bacterial, archaea, and fungal species, plus viruses and protists [1]. Despite this abundance and the importance of soil microorganisms for key ecosystem functions, the diversity and structure of soil microbial communities remain poorly studied [3-5]. With the development of metagenomic techniques, efforts to characterize the full extent of microbial diversity, their role in a variety of global ecological functions including carbon balance, cycling of nutrients and promoting plant growth have been initiated [6-8].

Terrestrial plants experience complex interactions with microbes found immediately surrounding the root (rhizosphere) and inside of root tissues

(endosphere). The microbiomes in these root-associated environments are comprised of bacteria, fungi, and to a lesser extent archaea, each with potential beneficial, neutral or detrimental effects on hosts' growth and development [7–12]. A thorough understanding of these complex relationships requires knowledge of resident microbes and factors shaping their abundance and community structure.

Central Texas has a region of uplifted limestone, the Edwards Plateau, providing an island of Glen Rose soils that fostered the speciation of many organisms and forms one of North America's areas of endemism. The soils are extremely alkaline, requiring unexplored plant – microbe adaptations and novel biochemistry. The climate of central Texas has historically been extremely unpredictable. Few studies have examined bacterial and fungal root communities from the same host or genotype over time, and none have examined these relationships in plants endemic to the Edwards Plateau. Thus, a deeper analysis of root microbiome of plants endemic to the Canyonlands division of the BCP, and characteristic of the Vireo Preserve as a function of host and environmental factors is pivotal for expanding our understanding of the nature and function of these systems.

## Methods

### *Sample Collection and Research Sites*

Samples were collected from seven plant species: *Muhlenbergia reverchonii*, *Yucca rupicola*, *Sophora secundiflora*, *Juniperus ashei*, *Arbutus xalapensis*, *Nolina lindheimeri*, *Prosopis glandulosa* at environmentally distinct sites within Wild Basin Creative Research Center boundaries (Fig.1: sites 3, 5, 9, and 11). No samples were collected from the Vireo preserve in 2015. Bulk soil samples were collected from the soil surrounding the plant specimen that were not in direct contact with its roots. Neighboring soil was collected from soil loosely adherent to plant roots, and was gently shaken off of specimens roots and collected. Root samples were obtained by carefully excavating and tracing the roots back to the target plant to ensure the identity of the individual roots sampled and correspondence between the host genotype and root samples.

Tertiary fine roots were shaken and washed with 100 ml of 10 mM NaCl solution. The resultant wash was collected in 50 mL tubes, which was then defined as the rhizosphere sample. For endosphere samples, the surface of the same root samples were sterilized by rinsing root samples an additional 4 times with sterile distilled water. The roots with diameter 2 mm or less were then transferred to 50 ml centrifuge tubes and washed using 6.15% of NaOCl with 2 to 3 drops of Tween 20 per 100 ml for 3 min, 100% ethanol for 30 s, and again with 3% of H<sub>2</sub>O<sub>2</sub> for 30 s. These surface sterilized roots were then rewashed 3 additional times with sterilized distilled water. The sterility of the root surface was assessed by plating a subsample of surface disinfected root onto LB plates and incubating the plate overnight at 30°C. If contamination was found the procedure above was repeated. These surface sterilized root samples constitute endophyte samples.

### *Microbial DNA Isolation and 454 pyrosequencing*

For rhizosphere samples, 2.0 ml of rhizosphere material were pelleted via centrifugation. The resultant pellet was then used for extractions using a PowerSoil DNA

extraction kit (MoBio, Carlsbad, CA). For endophyte samples, the surface sterilized roots were chopped into 1 mm sections, divided into 50 mg subsamples, sonicated, and total DNA was extracted using PowerPlant DNA isolation kit (MoBio, Carlsbad, CA) with the following modifications relative to manufacturer's instruction. We added 50 ul of 10% cetyltrimethylammonium bromide to each lysis tube containing the lysis solution and roots to enhance plant cell lysis, followed by three freeze-thaw cycles (80°C/65°C; 10 min each). Three subsamples were then concentrated and combined into a single 50 ul extraction. PCR of bacterial and fungal rDNA domains was conducted with pairs of fungal 18S or bacterial 16S primers.

16S rDNA genes were amplified using: 515F:GTGCCAGCMGCCGCGGTAA;

806R:GGA CTACHVGGGTWCTAAT

18S rDNA genes were amplified using: SSUfungiF: TGGAGGGCAAGTCTGGTG;

SSUfungiR:TCGGCATAGTTTATGGTTAAG

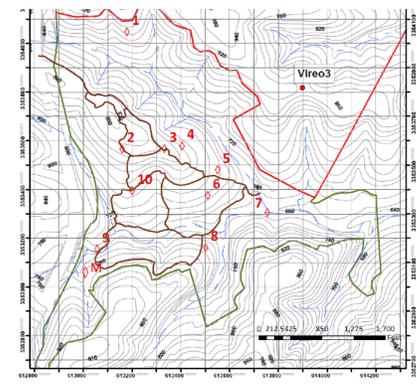
### Sequence Analyses

Sequences were submitted for sequencing, and will be analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software package (Caporaso, 2010a).

Sequences will be removed if their lengths were shorter than 200 nt, their average quality score was <25, and they contained ambiguous bases, primer mismatches, homopolymer runs in excess of six bases or error in barcodes. Filtering of noisy sequences, chimera checking and operational taxonomic unit (OTU) picking will be performed using the usearch series of scripts. De novo and reference-based chimera checking will be performed and sequences that were characterized as chimeric by both methods were removed. Sequences will be chimera-checked and clustered into OTUs with a minimum pair-wise identity of 97%. Each cluster will be represented by its most abundant sequence. Representative OTUs sequences will be aligned to the Greengenes database (13) using the PyNAST algorithm (minimum percent identity was set at 80%) (14). A phylogenetic tree will be built using FastTree (15). Taxonomy was subsequently assigned to each representative OTUs using the Greengenes database classifier with a minimum support threshold of 80%. Summary plots will then be generated using the R statistical package, Phyloseq (16).

### Results

Plant and soil samples were collected at 5 sites within Wild Basin Creative Research Center (Fig. 1, Sites 3, 4, 5,9, 11).



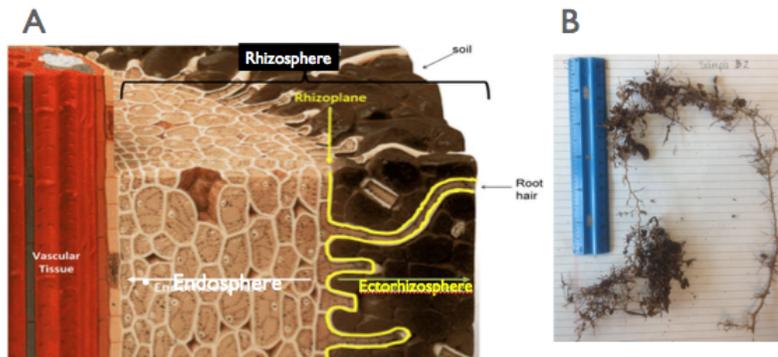
**Fig. 1** Wild Basin Wilderness Preserve. Plants were sampled from sites: 3,4,5,9,11.

Seven plant species were sampled from the following sites on two separate dates in this study (Table 1, Supplemental Table 1):

**Table 1: Plant Species Sampled Summer 2015**

Plant Species	Common Name	Site
<i>Arbutus xalapensis</i>	Texas madrone	9
<i>Juniperus ashei</i>	Ashe juniper	3, 11
<i>Muhlenbergia reverchonii</i>	Seep muhly	3,4,5
<i>Nolina lindheimeri</i>	Devils shoestring	3
<i>Prosopis glandulosa</i>	Honey mesquite	8
<i>Sophora secundiflora</i>	Mountain laurel	8
<i>Yucca rupicola</i>	Twistleaf yucca	3

For each plant sampled, a root segment of ~5 cm in length and 0.5–3 mm in diameter was collected near the base of the plant, along with any adherent soil particles. Bulk soil samples across each of the sites were also be collected by removing the top organic layer and sampling 2-3cm below that. All samples were returned to the lab and either frozen (-80 °C before DNA extraction, or DNA prepared the same day. DNA was isolated from four domains for each plant collected (Fig. 2): bulk soil (soil not associated directly from plants but from same sample site); neighboring soil (soil loosely adherent to plant root); rhizosphere (soil directly adherent to root, 2-3mm); endosphere (within root tissue itself).

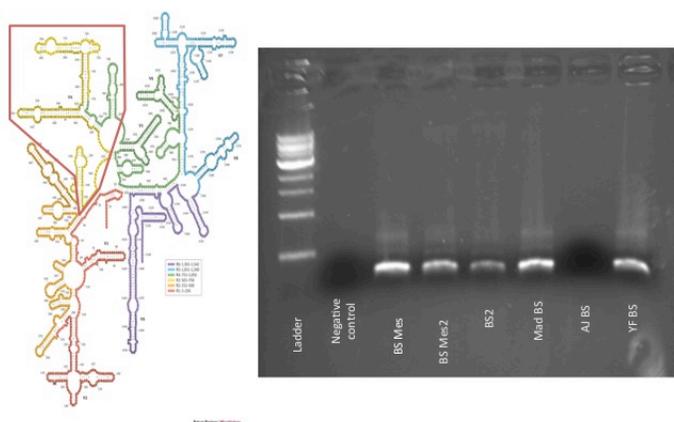


**Fig. 2. Soil Domains Sampled.** (A) DNA was isolated from the endosphere, ectorrhizosphere (rhizosphere), and the neighboring and bulk soil (not shown). (B) Image of *J. asheii* root sampled.

Fractionation of each plant sample into the constituent fractions (bulk, neighboring, rhizosphere and endosphere), and subsequent DNA isolation from each fraction followed standard protocols (Materials and Methods).

#### DNA Amplification

To validate samples for sequencing, rDNA sequences were amplified by using bacterial and fungal primers. The results for bulk soil samples show successful PCR amplification of bacterial DNA (V3-V4 domain) from *Muhlenbergia reverchonii* samples (Fig 3).



**Fig. 3** *Muhlenbergia reverchonii* PCR Analysis. (A) 16S rDNA domains. V3-V4 domain outlined in red. (B) PCR results using bacterial 16S primers.

Based on successful test amplification of bacterial and fungal rDNA domains from each plant sample DNA samples were submitted for microbiome sequencing. DNA sequence data will be analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software package (Caporaso, 2010a).

### ***Bacterial Microbiome Sequence Analysis of Samples Collected Summer 2014***

Preliminary analyses of the microbiome sequence data obtained from the plants sampled summer 2014 was carried out using the QIIME analysis pipeline during Summer 2015.

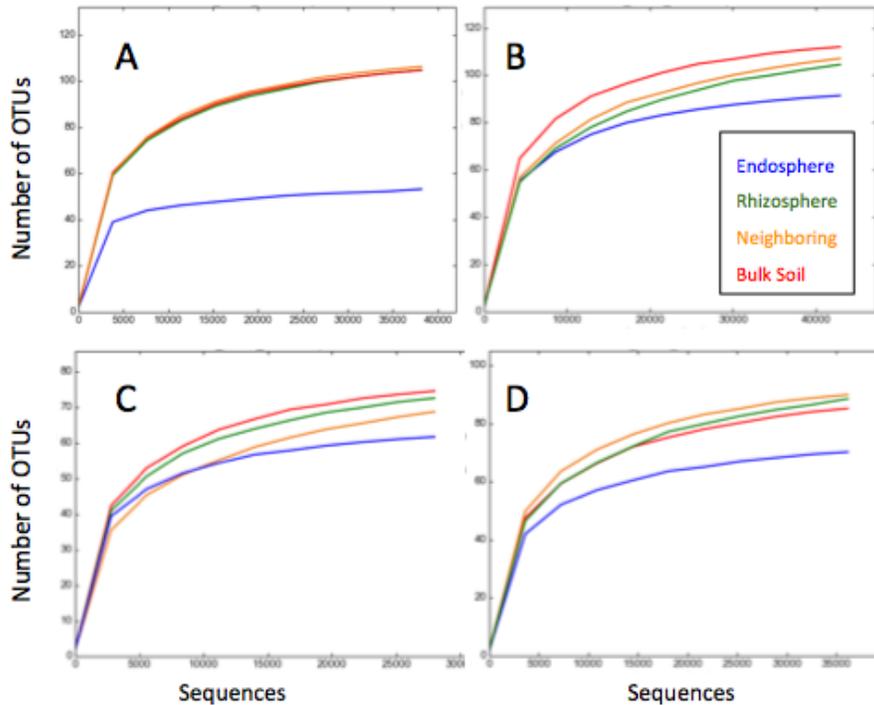
**Table 2:** Plant Species Sampled Summer 2014

Site	Species	Common Name
3, 4	<i>Muhlenbergia reverchonii</i>	Seep Muhly
3, 5, VP	<i>Carex planostachys</i>	Cedar Sedge
4, 11	<i>Schizachyrium scoparium</i>	Blue-Stem Grass
3, 4	<i>Juniperus ashei</i>	Ashe Juniper

### ***Community Sampling***

Rarefaction curves to 30-40,000 reads in each soil fraction indicated that, even at 40,000 reads, we were not capturing the entire community in any soil sample (Fig. 4).

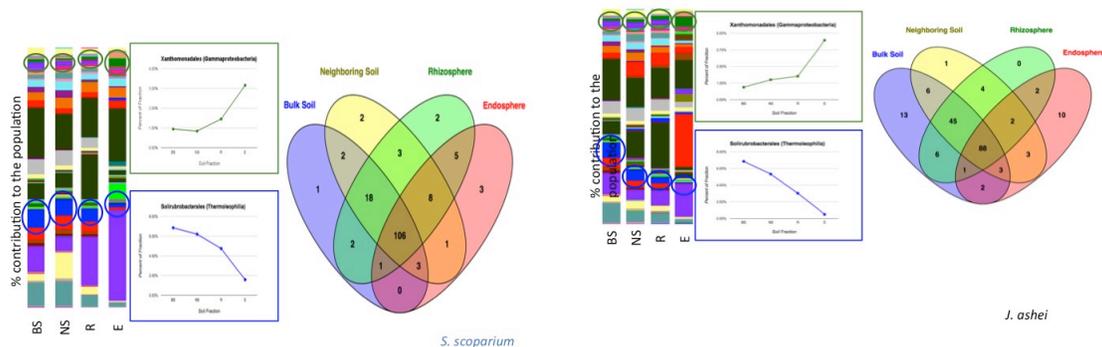
Consequently, the total number of OTUs we report for our soils may be lower than the true microbial diversity in soils.

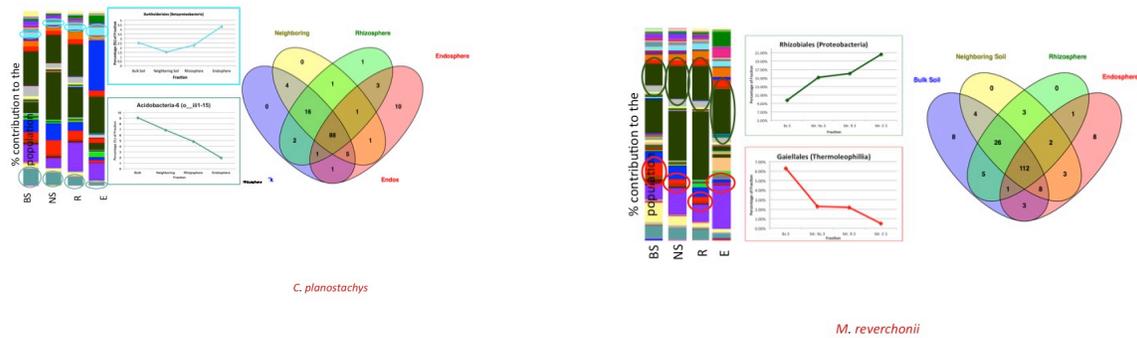


**Fig. 4 Sequencing statistics.** Rarefaction curves to 40,000 sequences for cumulative reads. A) *J. ashei*, B) *M. reverchoni*, C) *C. planostachys*, D) *S. scoparium*

### Alpha Diversity

Within-sample bacterial diversity for each plant and each sub-fractions was estimated using QIIME (Fig. 5) and stacked histograms and venn plots generated. Depicted at the *order* level, the stacked histograms for all four species indicate diverse bacterial populations in all fractions (bulk, neighboring, rhizosphere, endosphere). For each species we find taxa that are enriched or depleted within the endosphere fractions compared to bulk soil (Fig 5. Plots). The occupancies of each taxa within the four fractions are visualized using the venn diagram. In *J. ashei*, preliminary analyses indicate 13 unique taxa in bulk soil, 1 in neighboring soil, none in rhizosphere, and 10 in endosphere.

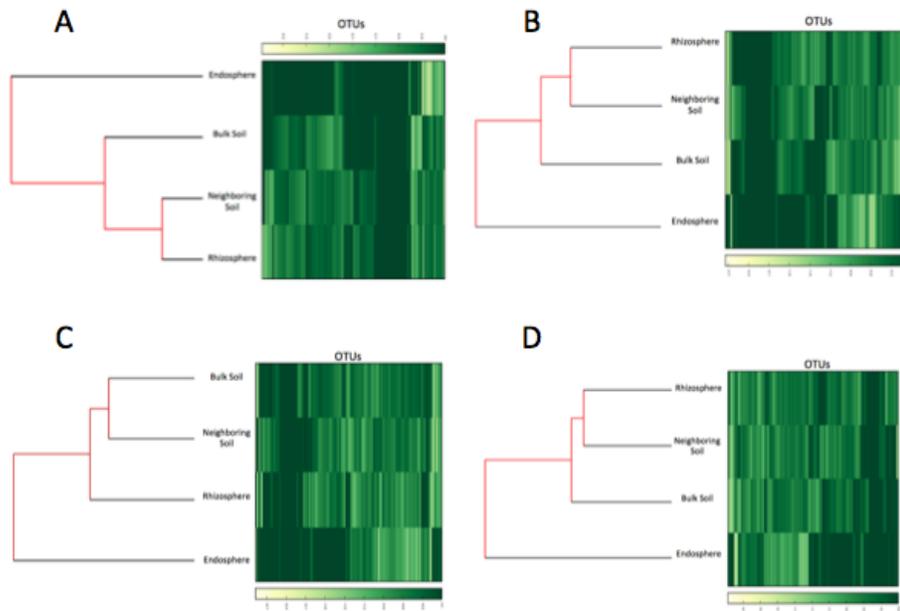




**Fig. 5** Alpha Diversity. A) *S. scoparium* B) *Juniperous ashei*; C) *C. planostachys*; D) *M. reverchonii*. BS: bulk soil, NS: neighboring soil, R: rhizosphere, E: endosphere

### Beta Diversity

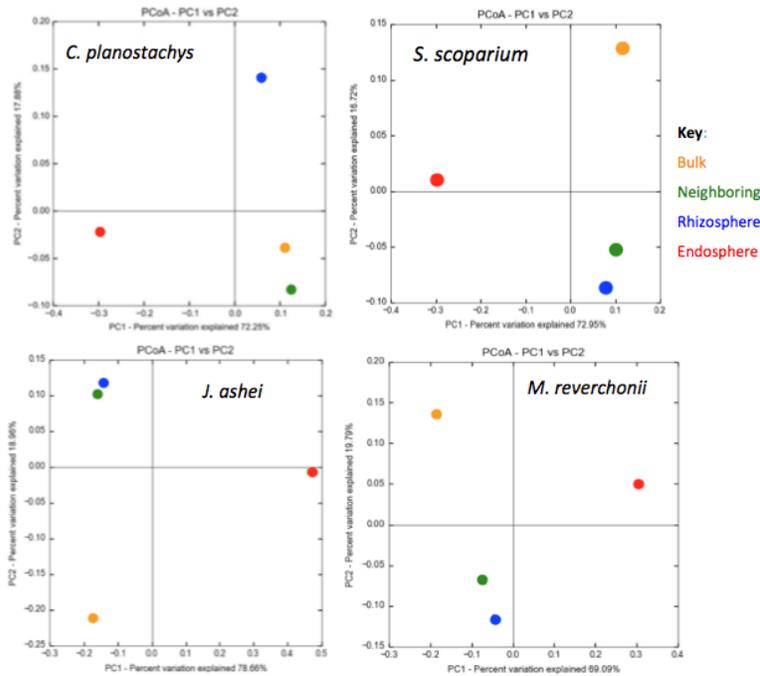
Community diversity between taxa (beta diversity) was computed using the QIIME workflow for bacteria (Fig 6). A preliminary analysis indicated that (1) rhizosphere and neighboring soil communities are most similar to one another for *J. ashei*, *S. scop*, *M. reverchonii*, however somewhat surprisingly, bulk soil communities appeared to be most similar to neighboring soil in *Carex*. The endosphere community appears to be the most unique for all species.



**Fig. 6** Beta Diversity Heatmaps. A) *J. ashei*, B) *M. reverchonii*, C) *C. planostachys*, D) *S. scoparium*

As a second approach to assess the bacterial beta diversity between the four fractions of each plant sampled principle component analyses was performed using the QIIME pipeline (Fig. 7). The 1<sup>st</sup> principle component for all species places endosphere into a group separate from bulk, neighboring and rhizosphere, perhaps indicating a ‘within-

plant' group, and 'external to' grouping. The 2<sup>nd</sup> principle component for three plants, *S. scoparium*, *J. ashei*, and *M. reverchonii* groups rhiosphere and neighboring together with bulk and endosphere forming separate groups. In contrast, for *C. planostachys* bulk and neighboring cluster together, with rhizosphere and endosphere forming separate groups.



**Fig. 7** Principle Coordinate Analyses A) *C. planostachys*; B) *S. scoparium*; C) *J. ashei*; D) *M. reverchonii*

### ***Fungal Microbiome Sequence Analysis of Samples Collected Summer 2014***

Analyses of the root-associated fungal microbiomes associated with plants collected during summer 2014 are currently in progress, and no QIIME analyses are available at this time.

### **Discussion and Conclusions**

Over the summer of 2015 osil samples associated with seven plant species at Wild Basin Creative Research Center were collected: *Muhlenbergia reverchonii*, *Yucca rupicola*, *Sophora secundiflora*, *Juniperus ashei*, *Arbutus xalapensis*, *Nolina lindheimeri*, *Prosopis glandulosa*. For each plant DNA from bulk soil not associated with plants was isolated for use as a control for fungal and bacterial populations present at each site. DNA was also prepared from three root-fractions: neighboring soil (loosely adherent to the root); rhizosphere (closely adherent to the root (2-3mm); and endosphere (within the root tissue itself). Bacterial and fungal root microbiomes associated with these species is currently in progress.

Sequence data obtained from bacterial (16S rDNA) for the four species sampled during Summer 2014 (*Muhlenbergia reverchonii*, *Carex planostachys*, *Schizachyrium scoparium*, and *Juniperus ashei*) was analyzed using QIIME. Preliminary analyses reveal: 1) diverse communities at the order level for all fractions in all plants sampled; 2)

potential recruitment of specific bacterial populations to rhizosphere and endosphere for each species; 3) endosphere communities are distinctly different from all others in all plants; 4) for three plants, *S. scoparium*, *J. ashei*, and *M. reverchonii* rhiosphere and neighboring together with bulk and endosphere forming separate groups. In contrast, for *C. planostachys* bulk and neighboring cluster together, with rhizosphere and endosphere forming separate groups.

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## Publications

Katrina Chuah, Andres Garza, Louise Gaunt, James Stewart, Jacquelyn Turcinovic, Margaret Walsh, Dr. Charles Hauser (2015) *Analysis of fungal and bacterial root microbiomes of plant communities at Wild Basin*. St. Edward's University School of Natural Sciences Lucian Symposium.

**Permits and Funding**

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