

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

Permit #: Hauser – 2014

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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, 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 soils are extremely alkaline, requiring unexplored adaptations and novel biochemistry. 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). 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, archaea) characteristic of Glen Rose Soils (bulk) present within BPC.
2. Characterize microbial communities (Fungal, eubacterial, archaea) 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, archaeal, 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 four plant species: *Muhlenbergia reverchonii*, *Carex planostachys*, *Schizachyrium scoparium*, *Juniperus ashei* at environmentally distinct sites: three were in the Wild Basin Wilderness Preserve (sites 3, 5, and 11) and one was located in the Vireo Nature Preserve (VP). Bulk soil samples were collected from the soil surrounding the plant specimen that were not in direct contact with its roots. Neighboring soil is loosely adherent to plant roots and was gently shaken off of specimens roots and collected. Plant roots were then collected so they could be washed to collect rhizosphere samples, and sonicated to collect endosphere samples.

We collected root samples by carefully excavating and tracing the roots back to the target plant to ensure identity of the individual roots sampled and correspondence between the host genotype and root samples. Tertiary fine roots shaken and washed with 100 ml of 10 mM NaCl solution to remove the adhering rhizosphere soil. 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 first root sample 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 H₂O₂ 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. For bacteria, the V3-V4 region of 16S rDNA was amplified.

Primer Sets

16S rDNA genes were amplified using: 515F:GTGCCAGCMGCCGCGGTAA;
806R:GGACTACHVGGGTWTCTAAT

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

Sequence Analyses

Sequences will be analyzed using the QIIME software package (Quantitative Insights into Microbial Ecology) using default parameters for each step (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 4 sites within Wild Basin Creative Research Center (Sites 3, 4, 5,11) and 1 site within the Vireo Preserve (Figure 1, Supplemental Table 1).

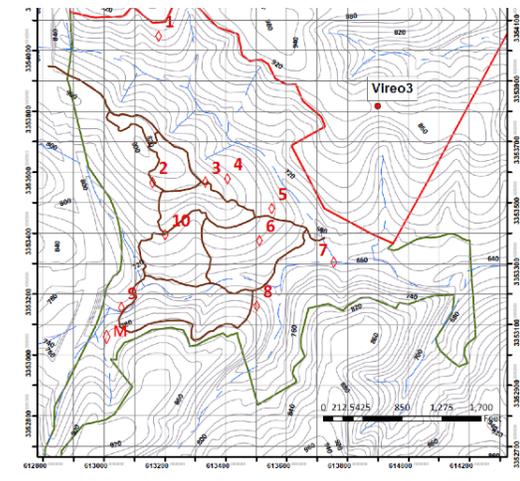


Fig. 1 Map of Soil Metagenomic Sampling Sites. Wild Basin Wilderness Preserve and Vireo Preserve Sites

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

Table 1: Plant Species Sampling Sites

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

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).

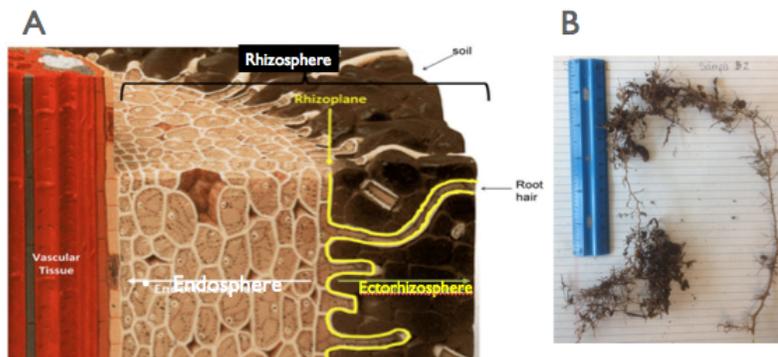


Fig. 2. Soil Domains Sampled. (A) DNA was isolated from the endosphere, ectorhizosphere (rhizosphere), and the neighboring and bulk soil (not shown). (B) Image of *J. ashei* root sampled.

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

DNA was isolated for plants as described above and prior to submission of samples for sequencing PCR amplification tests were conducted using both bacterial and fungal primers.

Carex planostachys (cedar sedge)

The results for cedar sedge (*Carex planostachys*) show successful PCR amplification of bacterial DNA (450nt band) from neighboring soil, endosphere and

rhizosphere samples (Fig 3B). In contrast, fungal rDNA (400 nt) has only been identified in one endosphere sample from the Vireo Preserve to date (Fig 3A).

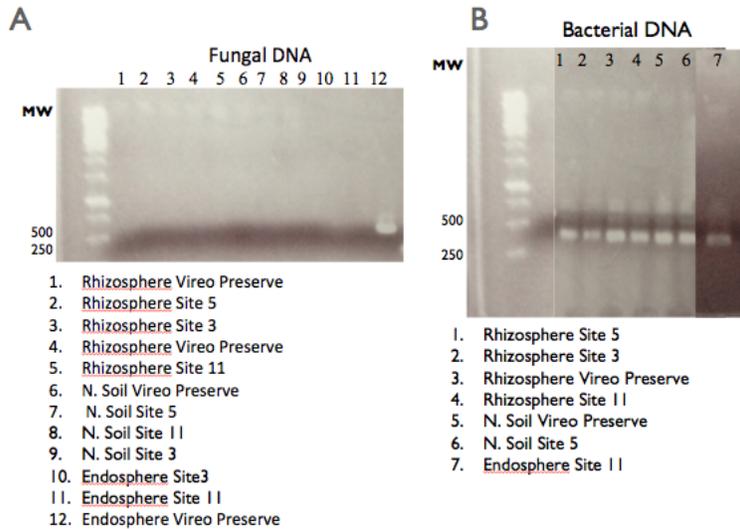


Fig. 3 *C. planostachys* PCR Analysis. (A) PCR results using fungal 18S primers. (B) PCR results using bacterial 16S primers. Sample keys (below).

Muhlenbergia reverchonii (Seep Muhly).

PCR amplification of fungal and bacterial DNA from *M. reverchonii* fractions has similarly been partially successful. Bacterial 16S rDNA has been successfully amplified from bulk soil, rhizosphere and endosphere samples, and fungal rDNA amplified from only rhizosphere samples. The results for amplification of fungal and bacterial rDNA from rhizosphere samples is shown in Fig. 4.

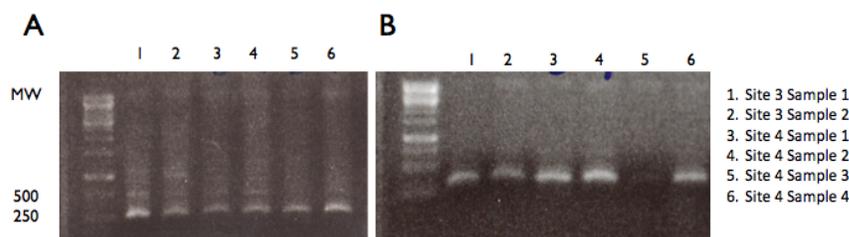


Fig. 4 *M. reverchonii* Rhizosphere PCR Analysis. (A) PCR results using fungal 18S primers and . (B) PCR results using bacterial 16S primers. Sample keys (right).

Shizachyrium scoparium (Little Bluestem)

Amplification of bacterial DNA from *S. scoparium* bulk soil, neighboring soil, rhizosphere and endosphere samples yielded a laddering set of products suggesting problems with the PCR reaction conditions (Fig. 5). Amplification using fungal primers from these samples is in progress.

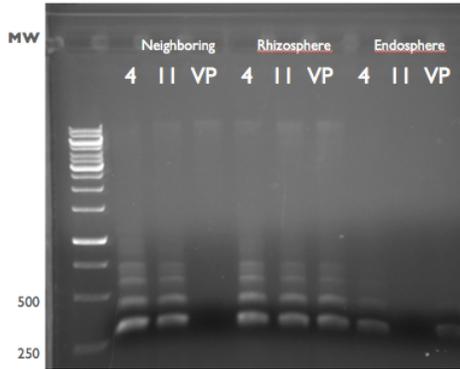


Fig. 5 *S. scoparium* Bacterial PCR Analysis. PCR results using bacterial 16S rDNA primers and neighboring, rhizosphere, and endosphere DNA samples. DNA isolated from plants collected at sites 4, 11, and vireo preserve (VP).

Juniperus ashei (Ashe Juniper)

Bacterial DNA has been successfully amplified from *J. ashei* bulk soil, neighboring soil, rhizosphere, and potentially from endosphere samples (Fig. 6.). Attempts to amplify fungal rDNA from these samples is in progress.

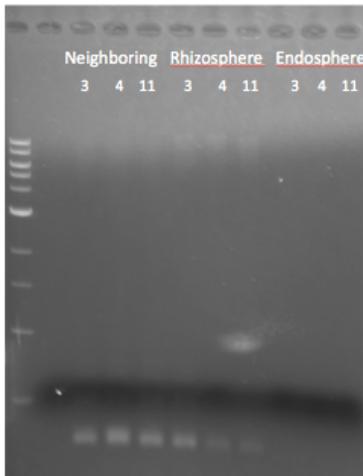


Fig. 6 *J. ashei* Bacterial PCR Analysis. PCR results using bacterial 16S rDNA primers and neighboring, rhizosphere, and endosphere DNA samples. DNA isolated from plants collected at sites 3, 4, 11.

Discussion and Conclusions

Over the summer of 2014 *Muhlenbergia reverchonii*, *Carex planostachys*, *Schizachyrium scoparium*, and *Juniperus ashei* plants were sampled from four distinct habitats within Wild Basin and the Vireo Preserve (Fig. 1, Supplemental Table 1). From these sites DNA from bulk soil not associated with plants was isolated for use as a control for fungal and bacterial populations present at each site. For each plant root sampled, DNA was isolated from three fractions: neighboring soil (loosely adherent to the root); rhizosphere (closely adherent to the root (2-3mm)); and endosphere (within the root tissue itself). Fungal and bacterial rDNA PCR analyses have been conducted on the majority of samples collected. The DNA quality, judged by A₂₆₀/A₂₈₀ ratios (Supplemental Table II), while variable should be of sufficient quality to obtain PCR products. Bacterial 16S rDNA PCR products have been obtained from the majority of fractions from each plant sample. Results from attempts to amplify Fungal 18S rDNA from the same DNA samples has yielded few positive results to date. This may indicate either a problem with the primers and/or PCR conditions employed, or an absence of fungal DNA in the samples. The latter condition is believed to be less likely, given our previous ability to amplify fungal, bacterial and bacterial DNA from bulk soil from soils samples obtained at Wild Basin (Hauser, 2013 Report). Attempts to refine PCR conditions, and fungal primers employed are in progress. Backup plant samples have been stored in freezer (-80°C) and are available for DNA isolation if required.

DNA samples for which we are confident that bacterial and/or fungal DNA can be amplified will be sent for 454 sequencing (MrDNA) Fall 2015, and the sequence data obtained analyzed Spring 2015. Upon obtaining DNA sequences, identification of fungal and bacterial communities present in each sample will be identified as described (Materials and Methods) and comparisons made between bulk, neighboring, rhizosphere and endosphere communities.

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Publications

Dylan Fox¹, Frank Garza¹, Dylan Sosa¹, Margaret Walsh¹, Lisa O'Donnell², Jim O'Donnell², Charles Hauser¹, “Analysis of Fungal and Bacterial root microbiomes of plant communities at wild basin”, Summer Research Symposium Symposium, St. Edward's University, June 26, 2014, ¹St. Edward's University Austin TX, ²City of Austin, Sr. Biologist, Balcones Canyonlands Preserve

Permits and Funding

This project was conducted under permits to Charles Hauser: City of Austin Permit (Hauser, 2014) and Travis County, Vireo Preserve Permit (Hauser, 2014). Funding for this project was made possible by: W.M. Keck Foundation, NSF STEP Grant #0969153, and TG Public Health Benefit Grant Program.

Supplemental Table I Plants and Sampling Sites

Date	Time	Site #	Plant Name	Plant ID	GPS Location	Altitude	Soil/Location Description
5/28/14	10:08	3	Muhlenbergia reverchonii	mr1	030.30830° / 097.82142°	838 ft	very rocky, dry, medium brown color, low slope, with steps, open grassland, scarce canopy
5/28/14	10:15	3	Muhlenbergia reverchonii	mr2	030.30835° / 097.82141°	856 ft	
5/28/14	10:31	4	Muhlenbergia reverchonii	mr3	030.30899° / 097.82116°	812 ft	very rocky, very wet, grey clay, steep sloping stair steps, mostly grasses, few woody plants, scarce canopy
5/28/14	10:39	4	Muhlenbergia reverchonii	mr4	030.30911° / 097.82124°	861 ft	
5/28/14	10:53	5	Carex planostachys	cp1	030.30791° / 097.81912°	779 ft	dark brown color, moist soil, lots of canopy coverage from cedar, low slope
5/28/14	11:00	5	Carex planostachys	cp2	030.30397° / 097.82453°	808 ft	
5/28/14	9:31	11	Carex planostachys	cp3	030.30665° / 097.81831°	620 ft	flat grassland, rich dark soil, rocky, furthest down wild basin, moist soils, surrounding canopy
5/28/14	8:59	11	Schizachyrium scoparium	sc1	30.30684° / 097.81797°	751 ft	
5/28/14	9:22	11	Schizachyrium scoparium	sc2	30.30667° / 097.81817°	673 ft	
5/28/14	9:07	11	Juniperus ashei	ja1	030.30685° / 097.81809°	686 ft	
5/28/14	9:50	11	Juniperus ashei	ja2	030.30681° / 097.81788°	571 ft	
5/28/14		3	Juniperus ashei	ja3			
6/4/14	8:38	3	Muhlenbergia reverchonii	mr5	030.30836° / 097.82134°	852 ft	
6/4/14	8:51	3	Muhlenbergia reverchonii	mr6	030.30839° / 097.82146°	854 ft	Under E. Sumac
6/4/14	9:15	3	Muhlenbergia reverchonii	mr7	030.30101° / 097.82596°	848 ft	Under Ash Juniper
6/4/14	9:45	4	Muhlenbergia reverchonii	mr8	030.30890° / 097.82078°	787 ft	
6/4/14	9:11	3	Juniperus ashei	ja4	030.30938° / 097.82145°	857 ft	
6/4/14	9:28	3	Juniperus ashei	ja5	030.30831° / 097.82143°		w/ Curly Mubly
6/4/14	9:43	4	Juniperus ashei	ja6	030.30894° / 097.82077°	794 ft	juvenile
6/4/14		Vireo	Carex planostachys	cp4	030.31209° / 097.21773°		
6/4/14		3	Carex planostachys	cp5	030.30824° / 097.82123°		
6/4/14		5	Carex planostachys	cp6	030.30261° / 097.81812°		
6/4/14		11	Schizachyrium scoparium	sc3	030.30689° / 097.81785°		
6/4/14		Vireo	Schizachyrium scoparium	sc4	030.31205° / 097.81773°		
6/4/14		4	Schizachyrium scoparium	sc5	030.31205° / 097.81773°		

Supplemental Table II. DNA Quality

Date	Pooled Tube Name	Site Number	Plant Name	DNA concentration (ng/microliter)			DNA (A ₂₆₀ /A ₂₈₀)			GPS Location
				(NS)	(R)	(E)	(NS)	(R)	(E)	
5/28/14	CM NS, CM R, CM E	3	<i>Muhlenbergia reverchonii</i>	40.06	29.31	26.04	1.87	1.91	1.57	030.30830° / 097.82142°
5/28/14	CM NS, CM R, CM E	4	<i>Muhlenbergia reverchonii</i>	31.47	35.1	21.16	1.74	1.94	2.00	030.30899° / 097.82116°
6/4/14	Ced NS, Ced R, Ced E	3	<i>Carex planostachys</i>	37.35	10.26	9.01	1.89	2.09	1.98	030.30824° / 097.82123°
5/28/14	Ced NS, Ced R, Ced E	5	<i>Carex planostachys</i>	28.8	18.54	26.42	1.77	2.00	1.36	030.30791° / 097.81912°
5/28/14	Ced NS, Ced R, Ced E	11	<i>Carex planostachys</i>	18.97	3.6	37.37	2.04	1.96	1.74	030.30665° / 097.81831°
6/4/14	Ced NS, Ced R, Ced E	Vireo	<i>Carex planostachys</i>	131.53	16.46	10.57	1.89	1.74	1.43	030.31209° / 097.21773°
6/4/14	Blu NS, Blue R, Blu E	4	<i>Schizachyrium scoparium</i>	56.5	10.01	10.53	1.92	2.10	1.64	030.31205° / 097.81773°
5/28/14	Blu NS, Blue R, Blu E	11	<i>Schizachyrium scoparium</i>	8.73	37.06	18.18	2.32	1.99	1.70	30.30684° / 097.81797°
6/4/14	Blu NS, Blue R, Blu E	Vireo	<i>Schizachyrium scoparium</i>	64.79	32.3	9.51	1.87	2.00	1.46	030.31205° / 097.81773°
5/28/14	AJ NS, AJ R, AJ E	3	<i>Juniperus ashei</i>	10.5	20.58	50.99	1.68	1.84	1.51	030.30894° / 097.82077°
6/4/14	AJ NS, AJ R, AJ E	4	<i>Juniperus ashei</i>	53.03	30.13	9.49	1.89	1.62	1.68	030.30685° / 097.81809°
5/28/14	AJ NS, AJ R, AJ E	11	<i>Juniperus ashei</i>	16.3	13.57	9.03	1.92	1.72	1.50	030.30685° / 097.81809°
5/28/14	ES NS, ES R, ES E	3	<i>Rhus virens</i>	74.19	54.34	21.02	1.94	1.88		

NS: Neighboring soil; R: Rhizosphere; E: Endosphere