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Comparison of microbial communities on roots, ceilings and floors of two lava tube caves in New Mexico

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Comparison of Microbial Communities on Roots, Ceilings and Floors of Two Lava Tube Caves in New Mexico

BY

Jessica Snider

Bachelors of Science, Biology, Texas State University, 1999

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master of Science

Biology

The University of New Mexico
Albuquerque, New Mexico

July, 2010

DEDICATION

I would like to dedicate this work to the four most important people of my graduate school life: my mentor Diana Northup, my best friend Brittany Barker, my mom Jo and my boyfriend, Yi-chun. To be a mentor, friend, parent or partner to a graduate student is hard under any circumstances, but it is a completely different thing when that graduate student's health is slowly failing in front of you and the very thing you are mentoring them on is what is hurting them. Diana, Brittany, Mom and Yi-Chun had to do this, and somehow, they always had the wisdom to know when to back off and let me do what I thought needed to be done, and when to step in and help, when to tell me "Come on, keep going, you can do this," and when to ask if what I was doing was really worth the price I would have to pay. I wish I could say I had the wisdom to always listen to them, but I do not think I did. The true horror of chronic illnesses like the kind that I have is that they sneak in and steal your dreams, your hopes, your hobbies, your passions, your goals and your future, little bit by little bit, until you finally just start to give up. Diana, Brittany, Mom and Yi-Chun were always there to remind me that my disorder had never stolen my future; it may have changed my goals, shifted how I would achieve my dreams, but I never lost anything. No matter how bad is all got, because of them, I always found the strength to get up again and keep fighting, and because of them, I always will.

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Finally, I would like a to thank all my friends and family who agreed to be field help, including Mary, Elijah, Jason, Cameron, Penny, Ken, Brittany, Yi-Chun, MJ and the members of the Sandia Grotto.

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ABSTRACT OF THESIS

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ABSTRACT

Lava tube caves possess a rich and patchy mosaic of nutrients and water that fosters a wide variety of microbial life. Water percolating past vegetation and through surface soils introduces dissolved carbon and nitrogen into caves. Roots provide even higher levels of carbon, nitrogen and microorganisms as they grow into the cave environment from surface vegetation. I hypothesized that this mosaic of nutrients and energy resources has a direct effect on the distribution and composition of microbial communities within caves. To test this hypothesis, I selected two lava tube caves in New Mexico, USA and identified six zones consisting of ceiling and floor sampling sites in areas with: (1) active root growth and active water percolation; (2) active water percolation from the surface, but no root growth and; (3) neither roots nor water. After determining percent carbon and nitrogen for each of the sites, I compared the results using ANOVA. I constructed 12 clone libraries using 16S rRNA-based culture-independent methods. Closest relatives were determined using the NCBI BLAST database and phylogenetic trees were constructed in MEGA 4. Diversity and community

composition were compared using *mothur*, UniFrac and traditional statistics. Roots formed zones of isolated, increased carbon and nitrogen, but water percolating into caves without the presence of roots did not bring significantly more carbon or nitrogen into lava tubes. The microbial community compositions of the six zones were different from each other at the 97% OTU level, but were similar in diversity, 'phylum' level distribution and relatedness to other bacteria found in other soil, rhizosphere and cave environments. This study has numerous management implications, including highlighting the importance of roots and percolating water within the cave environment and therefore, the need to protect surface vegetation above caves. In addition, given that roots represent a conduit from the surface into the cave, this study shows the importance of native vegetation above critical cave habitats to preserve cave nutrient inputs and endemic microbial communities.

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Introduction

Lava tubes, a type of cave found in volcanic deposits worldwide, have roofs a few meters thick to a maximum thickness of 10 meters (Palmer, 2007). They are within the root zones of numerous woody plant species, which have an average rooting depth of 6.8 meters (Canadell et al., 1996; Schenk and Jackson, 2002). As expected, roots have been found growing into caves in numerous locations, including the USA, Mexico, Japan, Azores, Canary Islands and Korea (Oromi and Martin, 1992). These roots, along with water percolating from the surface, bring carbon (C) and nitrogen (N) into lava tubes and are an important food source to numerous unique troglobites (Gillieson, 1996; Howarth et al., 2007). However, few studies have examined how roots affect the microbial communities and ecology of a lava tube.

As far as the plant is concerned, roots have three main purposes: (1) anchoring the plant to the substrate; (2) taking up water and nutrients from the soil and; (3) storing resources between uptake and consumption (Gregory, 2006; Raven and Edwards, 2001). Although most of the transport of nutrients and water in roots is from deeper soils up to aboveground plant parts, plant roots can also transport nutrients and water into deeper or intermediate soil levels (Jobbágy and Jackson, 2001; McCulley et al., 2004; Schenk and Jackson, 2002). Most woody plants produce sizable amounts of root exudates, which are organic compounds secreted by the root to influence the microbial communities of the rhizosphere (Inderjit and Weston, 2003). Additionally, as roots grow, the root cap sloughs off cells and the roots will produce fine roots, which explore soils and increase surface area available for absorption. As these fine roots grow and then die off, they are

added to the soil or cave C load as fine root turnover, which can account for several hundred g of C m^{-2} $year^{-1}$ (Lauenroth and Gill, 2003; Phillips et al., 2009 Raven and Edwards, 2001). New roots may unintentionally release up to 2% of their total sugars and other nutrients at areas of new growth since the plasma membranes of newly grown phloem cells tend to be porous (Farrar et al., 2003). The transported nutrients and the plant roots themselves impact the invertebrate and microbial communities of lava tubes. Troglobitic isopods in Hawaiian and Australian lava tubes have been found to feed directly on the phloem and xylem of plant roots (Gillieson, 1996; Howarth, 1972; Howarth et al., 2007; Oromi and Martin, 1992).

Additionally, microorganisms living within the rhizosphere are mechanically carried into the lava tube cave with root growth, where they may remain associated with the rhizosphere or spread throughout the cave environment. Since the rhizosphere microorganisms are immigrants into the cave ecosystem, they may represent a seeding source of microorganisms that adapt to the cave over time. As the mycorrhizal fungi and bacteria die, they serve as a food source to invertebrates and other microbes in the cave ecosystem (Jasinska et al., 1996; Simon and Benfield, 2001). A limited number of studies have looked at how roots affect lava tube environments (Oromi and Martin, 1992; Gillieson, 1996; Howarth, 1972; Howarth et al., 2007), but no studies have looked at microorganisms that are found to be associated with roots growing into lava tubes, or shallow caves, or how these communities differ from the microbial mats growing on the walls of the cave environment.

Sinking streams or water percolating down from the surface bring nutrients into

caves. Simon et al. (2003, 2007) found that sinking streams in a Virginia cave were N-rich but C-limited and that they brought in 7.67 mg/L of total organic C (TOC), while water percolating into the cave from the epikarst, the karst layer above the cave, had a TOC of 1.1 mg/L. Arid land cave systems rarely have sinking streams, but percolation does occur and may collect to form pools in the cave. TOC levels in arid land caves have been found to range from 0.02 mg/L TOC (Barton and Jurado, 2007) to 1.58 mg/L in pools (Northup et al., 1994) and 1.3 mg/L in seeping water (Levy 2007). Nutrients in the water percolating down into the cave can be deposited onto cave walls and floors, while C, N and other nutrients are also found as part of deep soil layers and bedrock (>1 m; McCulley et al., 2004). Northup et al. (2003) found 0.002-0.103% C and 0.01-0.04% N in the soils and ferromanganese crusts of Lechuguilla Cave in New Mexico. These nutrients, although limited in amount, can affect the location and composition of microbial communities present in cave, lava tube, or deep soil environments.

Several previous studies have begun to elucidate the microbial communities of limestone and lava tube caves. Both heterotrophic (Ikner et al., 2007; Pašić et al., 2009; Portillo et al., 2008; Portillo and Gonzales, 2009) and chemolithotrophic (Engel et al., 2004; Macalady et al., 2007; Northup et al., 2003; Northup and Lavoie, 2001; Sarbu, 1996) microbial communities have been identified in limestone caves associated with various available nutrient sources. Extensive microbial mats, known as "lava tube slimes," are commonly found in lava tube caves (Northup et al., 2008; Northup and Welbourn, 1997; Moya et al., 2009; Garcia et al., 2009; Snider et al., 2009) and were originally believed to consist of fungi, aerobic bacteria and *Actinobacteria* (Staley and

Crawford, 1975; Stoner and Howarth, 1981). The microbial communities of these mats are highly diverse, with representatives from 15 bacterial phyla and both heterotrophic and chemolithotrophic bacterial metabolisms present (Garcia et al., 2009; Hathaway, 2010; Northup et al., 2008; Moya et al., 2009; Snider et al., 2009). Although recent studies have assessed the effects of geography, mat color, average surface rainfall and other abiotic factors on microbial community composition of the "lava tube slime (Hathaway, 2010)," this study is the first to investigate the source and effect of nutrient availability in lava tubes, particularly the effect of tree roots on microbial communities in lava tubes.

Life in all environments is controlled in large part by the availability of water and nutrients. Plant roots entering caves will augment the available nutrients and microbial communities, creating a mosaic of nutrient availability, and may have a direct effect on the microbial community distribution and composition. Surface water moving through plant and soil layers and then into the caves will do much the same, only to a lower extent. I hypothesize that areas or zones with exposure to root growth and water percolation will have higher amounts of: (a) C and N, (b) higher microbial community diversity, (c) higher percentage of clones from phyla commonly found in other root and soil environments, such as *Alphaproteobacteria* and *Bacteroidetes,* (d) higher percentage of clones related to other soil or root-related bacteria and (e) microbial communities that are statistically significantly different from each other.

To test these hypotheses, I selected two lava tubes located in the El Malpais National Monument, New Mexico, with active and extensive root growth and defined the

following six zones related to root abundance and water content: (a) ceiling and floor zones with exposure to macroscopic roots and water flow from the surface; (b) ceiling and floor zones with macroscopic evidence of water flow from the surface, but no roots, and; (c) ceiling and floor zones with no macroscopic evidence of water flow or root activity. Total percent C and N and culture-independent studies of the microbial communities at each of these zones were conducted.

This study is the first to quantify C and N present at various locations within lava tube cave systems and to investigate their effects on the microbial communities of the caves. This study will highlight that roots represent a conduit from the surface into the cave, suggesting that cave management should include conservation of the vegetation above important cave locations. Numerous bacteria found in lava tubes, especially in the phylum *Actinobacteria*, are a potential source of novel antibiotics and other medicines. This study will document the presence of numerous novel possible antibiotic-producing *Actinobacteria* and other previously unknown bacteria. Finally, since caves represent voids in subsurface bedrock, this study should increase understanding of lava tubes and lava subsurface environments in general.

Materials and Methods

Cave Description and Sample Collection

Two lava tube caves, Roots Galore Cave and Pahoehoe Cave, located in the El Malpais National Monument in northwestern New Mexico were selected as collection sites because of their abundant root growth and controlled access by permit. Both caves

are at an elevation of 2265 m and are 0.32 km apart. The surface vegetation is composed of Ponderosa pine (*Pinus ponderosa)* and Great Basin desert shrubs. The site receives an average annual rainfall of 381 mm (www.nps.gov/elma/index.htm). Root growth in both caves is believed to be that of *Pinus ponderosa*, which has a reported average maximum rooting depth of 3.5 m (Canadell et al., 1996) and fine root turnover C inputs values of 115 to 290 g C m^{-2} $year^{-1}$ (Phillips, 2009). Aerial root growth in both caves extends approximately 0.33 m into the cave environment from the ceiling and is covered with white fungal and bacterial mats (Figure 1). Pahoehoe Cave is 200.79 m long and has a summer temperature 14.6°C, while Roots Galore Cave is 52.55 m long with a summer temperature of 15.4°C and a winter temperature of 9.6°C.

Triplicate root samples from each cave were collected aseptically from active root growth near the root apex during June and July of 2006 and 2009. Triplicate samples of wet and dry rocks and soils (approximately 2 g) were collected from the walls and floors of each cave at least 2 m from any noticeable root growth. Wet and dry samples were separated from each other by at least 0.33 m and corresponding floor samples were collected directly below the ceiling sites. All samples were collected aseptically using a sterile cold chisel or sterile scoopula, under a National Park Service collecting permit. Sampling sites are shown in Figure 2. Upon collection, the samples for DNA analysis for each zone were covered in sucrose lysis buffer (SLB) to ensure preservation of the DNA (Giovannoni et al., 1990), while nutrient samples were collected aseptically and stored on site in dry tubes or sterile Whirl-paks (Nasco, Wisconsin, USA). All samples were then transported on ice back to the laboratory at the Department of Biology, University of

New Mexico (UNM), Albuquerque, NM and DNA samples were stored at -80°C until DNA extraction could be performed. Nutrient samples were stored at 4°C and processed within three days of collection.

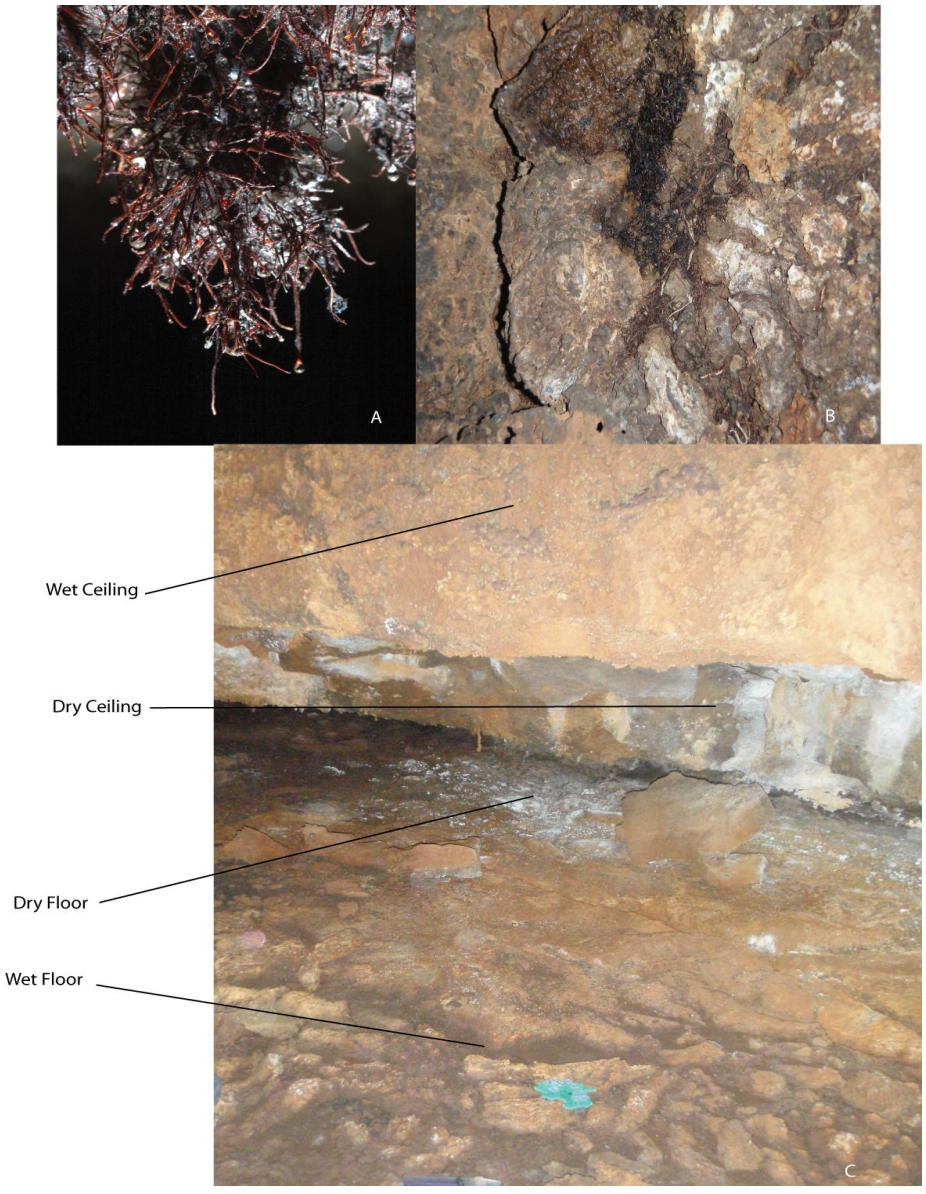


Figure 1: Photos of sampling locations in Roots Galore Cave. Photos show Root Zone (A), Root Floor Zone (B), Wet Ceiling Zone (C), Wet Floor Zone (C), Dry Ceiling Zone (C) and Dry Floor Zone (C).

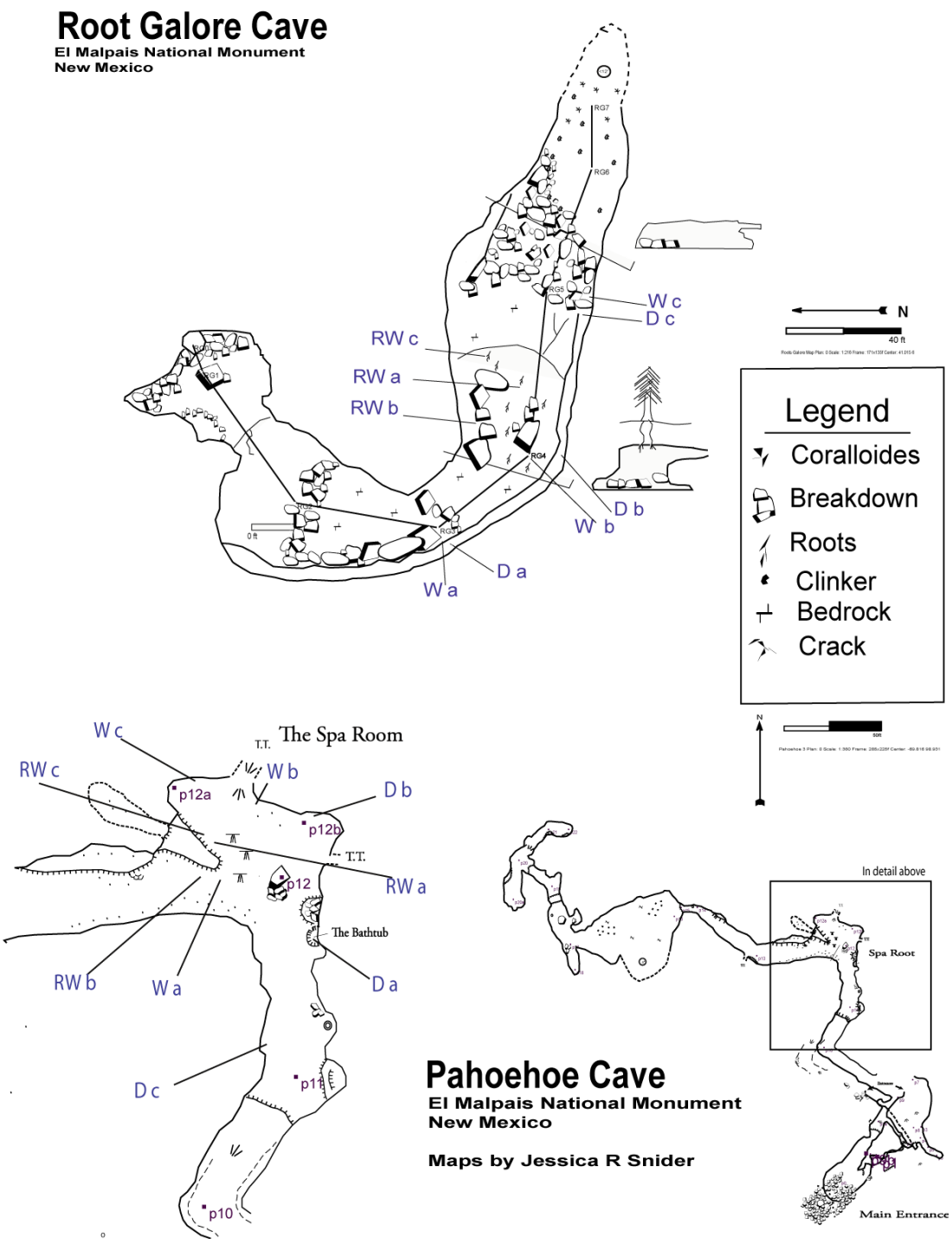


Figure 2: Map of sampling locations in Roots Galore and Pahoehoe Caves, El Malpais National Monument, New Mexico, USA. RW, W and D represent Root, Wet and Dry zones while a, b, and c identifies subsample.

Nutrient Analyses

Dry samples collected for nutrient analysis were desiccated, ground, and inorganic C was removed by HCl fumigation. Percent N and C were determined by high temperature combustion; the resulting gases were eluted on a gas chromatography column, detected by thermal conductivity and integrated to yield carbon and nitrogen content. Analysis was performed on a ThermoQuest CE Instruments NC2100 Elemental Analyzer (ThermoQuest Italia Sp.A., Rodano, Italy, Pella, 1990). Soil extractable nitrogen was determined by extraction with 2N KCl followed by analysis for NH_4-N using method 98-70W (1a), 4500-NH_3-G (2) and NO_3-N using method 100-70W (1b), 4500-NO_3-F (2), on a Technicon AutoAnalyzer II (Mulvanery, 1996). All analyses were completed at the UNM Biology Annex Labs.

Total percent C and N from both caves was averaged together for each zone and checked for normality using an Anderson-Darling test of Normality and Bartlett's F-test for Equal Variance. Since the data was not normally distributed, averaged sample amounts were compared using a non-parametric Mann-Whitney Two Sample test and Kruskal-Wallis ANOVA using Minitab v15. Results were considered statistically significant at the 95% ($P<0.05$) level.

DNA Extraction and PCR Amplification of 16S rRNA Genes

Triplicate samples for DNA extraction from each zone type (Root, Root Floor, Wet Ceiling, Wet Floor, Dry Ceiling and Dry Floor) were pooled to maximize the representation of indigenous members at each zone (Gomez-Alvarez et al., 2007) and

manually homogenized using sterile pliers and cold chisel. DNA was extracted and purified using the MoBio PowerSoil™ DNA Extraction Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's instructions, except for the use of a beat beating rather than vortexing and the elution in 25 µl of EB buffer. The 16S rRNA gene was amplified using universal bacterial primers 46 forward (5'-GCYTAAYACATGC AAGTCG-3') and 1409 reverse (5'-GTGACGGGCRGTGTGTRCAA-3') with an amplification reaction mixture that contained 1X PCR buffer with 1.5 mM Mg^{2+}, 5 mg bovine serum albumin (Boehringer- Mannheim), 200 mM (each) deoxynucleoside triphosphates, 100 pmol of each primer and 0.5 U of *Taq* polymerase (AmpliTaq LD; Perkin-Elmer) in a final reaction volume of 25 µl. DNA extraction and 16S rRNA amplification were done in triplicate to reduce extraction and PCR bias (Rastogi et al., 2010). Amplicons were cleaned and purified using a Qiagen PCR cleanup kit (Qiagen, Germantown, MD) and were cloned using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) with the pCR4.1 Vector. Early RG32 samples were sequenced using ABI PRISM Big Dye Terminator v1.1 sequencing kit (Perkin-Elmer, Foster City, CA) at the Molecular Biology Facility at the University of New Mexico, while RG22, RG23, RG24 RG25, RG28, later RG32, PH01, PH24, PH25 and PH27 samples were sent to Washington University Genome Sequencing Facility for sequencing with primers M13F and M13R. PH22 and PH26 were sequenced at University of Washington High-Throughput Sequencing Facility using primers T3 and T7. Sequences were assembled and edited with Sequencher 4.8 (Gene Codes, Ann Arbor, Michigan). Orientation of all sequences was checked using Orientation Checker (www.bioinformatics-toolkit.org) and

sequences were screened for possible chimeric artifacts using Mallard and Pintail (www.bioinformatics-toolkit.org; Ashelford et al., 2006). Closest relatives were selected using the NCBI BLAST (Altschul et al., 1997) and phylum grouping was determined using both the Ribosomal Database Project v10 Classifier (rdp.cme.msu.edu/classifier/classifier.jsp) and BLAST results combined. Alignments using full-length sequences were developed using GreenGenes (greengenes.lbl.gov/cgi-bin/nph-index.cgi; Hugenholtz, 2002) and manually refined using the BioEdit multiple sequence editor (www.mbio.ncsu.edu/BioEdit/ BioEdit.html) with consideration of primary and secondary structure. A Jukes-Cantor corrected distance matrix for each clone library was created by the DNADIST program (Felsenstein, 1990). Sequences with greater than 97% similarity were classified by DOTUR v1.53 (Schloss and Handelsman, 2005) as unique operational taxonomic units (OTUs) at the "species" level and were used to make a maximum parsimony phylogenetic tree with 1000 bootstrap replicates using MEGA 4.0. The maximum parsimony tree was obtained using the Close-Neighbor-Interchange algorithm and codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option) (Kumar et al., 2008; Tamura et al., 2007).

Statistical Analysis of Microbial Diversity

Rarefaction curves and Good's Coverage (C') were calculated to indicate how well each clone library captured microbial diversity at various taxonomic levels (Esty, 1986). OTUs were also classified at phylum, genus and species levels using sequence

similarities of 80%, 95% and 97% respectively, to assess coverage at each of these levels (Rastogi et al., 2010; Schloss and Handelsman, 2004). Bacterial diversity was statistically determined with the reciprocal of Simpson's index(1/D, simply called Simpson's index in this paper) and Shannon-Weaver index(H') using the default equations in DOTUR and *mothur* (Schloss and Handelsman, 2005; Schloss et al., 2009). A higher reading on a Simpson's and/or Shannon-Weaver index suggests a community with a greater number and a more even distribution of species (Gomez-Alvarez, 2007). Non-parametric Chao richness estimates were calculated by DOTUR. Venn diagrams were used to show common OTUs between different sites and Libshuff and UniFrac, two tests included in *mothur*, were performed to estimate differences in microbial community composition. Principal Coordinate Analyses (PCoA) and Jackknife clustering were completed in UniFrac to assess similarities in microbial communities throughout the cave (Lozupone et al., 2006; Lozupone and Knight, 2008). Percentages of clones with closest relatives related to cave, rhizosphere and soil associated microbes were complied for each zone and compared using two-proportion Fisher Exact tests in Minitab v15.

Results

Nutrients

The analyzed Root Zone had an average of 40.75% C, and the Root Floor Zones had an average of 5.86% C. Wet and Dry Floor Zones had 2.41% and 1.42% C, respectively, while both Ceiling Zones had less than 0.35% C (Table 1). Root Zones had significantly (P=0.000, KW-ANOVA) higher amounts of C than other samples, while the

zone Root Floor Zones were significantly lower (P=0.004, Mann-Whitney) in percent C than the actual roots, but were significantly higher (P=0.000, KW-ANOVA) than the other Ceiling or Floor Zones. While there was no significant difference between the percent C in the Wet and Dry Ceiling Zones and the Wet and Dry Floor Zones, there was a significant difference between the percent C in the Ceiling and Floor Zones (P=0.0003, Mann-Whitney).

Total percent N was much lower than percent C levels. Root Zones had, on average, 0.74% N, and Root Floor zones had an average of 0.40% N. Wet and Dry Floor Zones had 0.25% and 0.16% N, respectively, while both Ceiling Zones had less than 0.03% N (Table 1). Root Zones had significantly (P=0.000, KW-ANOVA; P=0.001 Mann-Whitney) higher percentage of N than other samples, while percent N in Root Floor Zones was significantly higher (P=0.0012, Mann-Whitney) than the other Ceiling or Floor Zones. While there is no significant difference between percent N in the Wet and Dry Ceiling Zones, there is a significant difference in percent N between the Wet and Dry Floor Zones (P=0.0.04, Mann-Whitney) and Ceiling and Floor Zones (P=0.0001, Mann-Whitney).

Since the roots and the floor below them represent such a huge load of C and N, further analysis was completed using just the Wet and Dry Ceiling and Floor Zones. C and N were related linearly (R^2=0.908, y=9.07x=0.023) to each other and fall out along this relationship according to Zone, with the Wet Floor Zones and one Dry Floor zone at the higher nutrient end and the Ceiling Zones at the lower nutrient end (Figure 3).

Table 1: Total organic carbon and nitrogen (%) in Pahoehoe and Roots Galore Caves at all six sampling zones. Results are averaged (N=6) and shown with averaged amount and standard deviation as calculated by Minitab v15.

Sampling Zone	Average Soil Carbon (%)	Average Soil Nitrogen (%)	Average C/N(Mol)
Root Ceiling	40.749 ± 16.76	0.741 ± 0.305	63
Root Floor	5.864 ± 1.35	0.400 ± 0.15	17.1
Wet Ceiling	0.358 ± 0.75	0.031 ± 0.02	13.5
Wet Floor	2.411 ± 0.72	0.253 ± 0.08	11.1
Dry Ceiling	0.228 ± 0.22	0.030 ± 0.02	9.5
Dry Floor	1.424 ± 0.85	0.165 ± 0.10	10.2

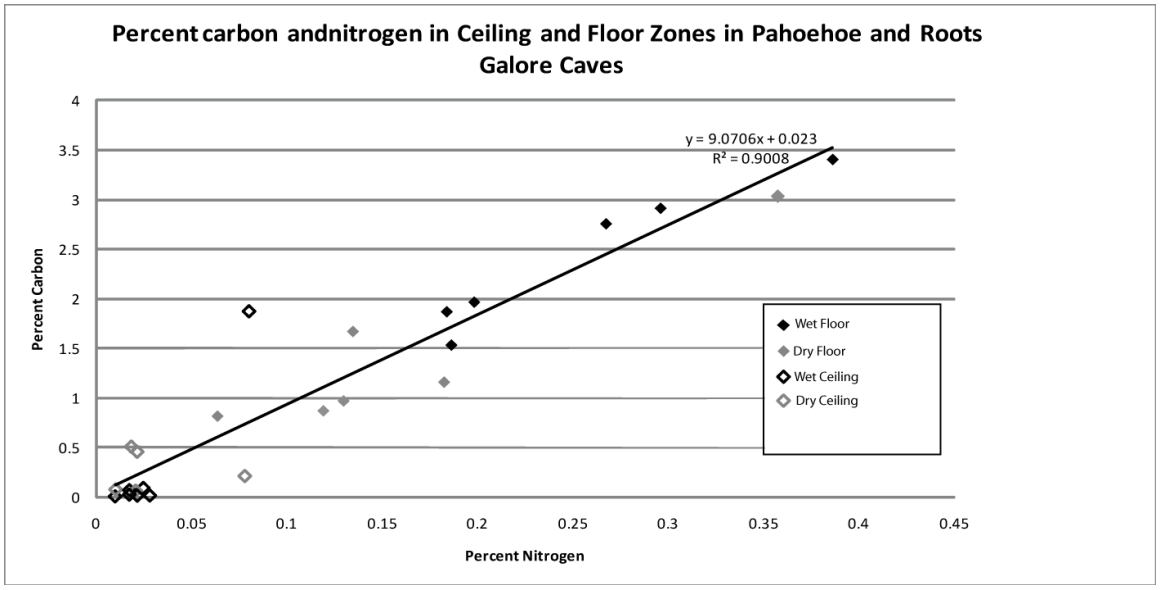


Figure 3: Percent carbon and nitrogen in Ceiling and Floor Zones in Pahoehoe and Roots Galore Caves.

Diversity of Microbial Communities

A total of 540 full full-length, non-chimeric sequences and 399 unique OTUs identified at the 97% sequence similarity were obtained from 12 clone libraries. Richness (S) of 118 OTUs were found on the Root Zone, 126 on the Wet Ceiling Zone, 116 on the Wet Floor Zone, 76 on the Dry Ceiling Zone and 75 on the Dry Floor Zone. Root Floor Zones had only 28 non-chimeric, full-length sequences recovered from a clone library of

over 300 attempted clones, suggesting problems with the PCR or cloning reaction. Because of this, clones from the Root Floor Zones were included in the phylogenetic trees, but not in the diversity, closest relatives or phylum analyses charts. Rarefaction curves for all zones show that saturation of sampling was not achieved at the 97%, but did show an indication of leveling off at the 80% (phylum) sequence similarity level (Figure 4). Coverage (C') values at the 97% sequence similarity level were 16%, 18%, 24%, 15.4% and 24.1% at the 97% sequence similarity level but were 69%, 79%, 88%, 72% and 80% at the 80% sequence similarity level for Root, Wet Ceiling, Wet Floor, Dry Ceiling and Dry Floor Zones, respectively (Table 2).

Simpson's and Shannon indices showed that diversity for Root Zone (H'=4.65, 1/D=178) was not significantly different than the diversity of the Wet Ceiling (H'=4.25, 1/D=65) and Dry Ceiling (H'=4.11, 1/D=195), but was different from the Wet Floor (H'=3.72, 1/D=23) and Dry Floor (H'=3.95, 1/D=98) at the 97% sequence similarity level (Table 2). Chao estimates also suggested similar patterns(Table 2, Figure 5).

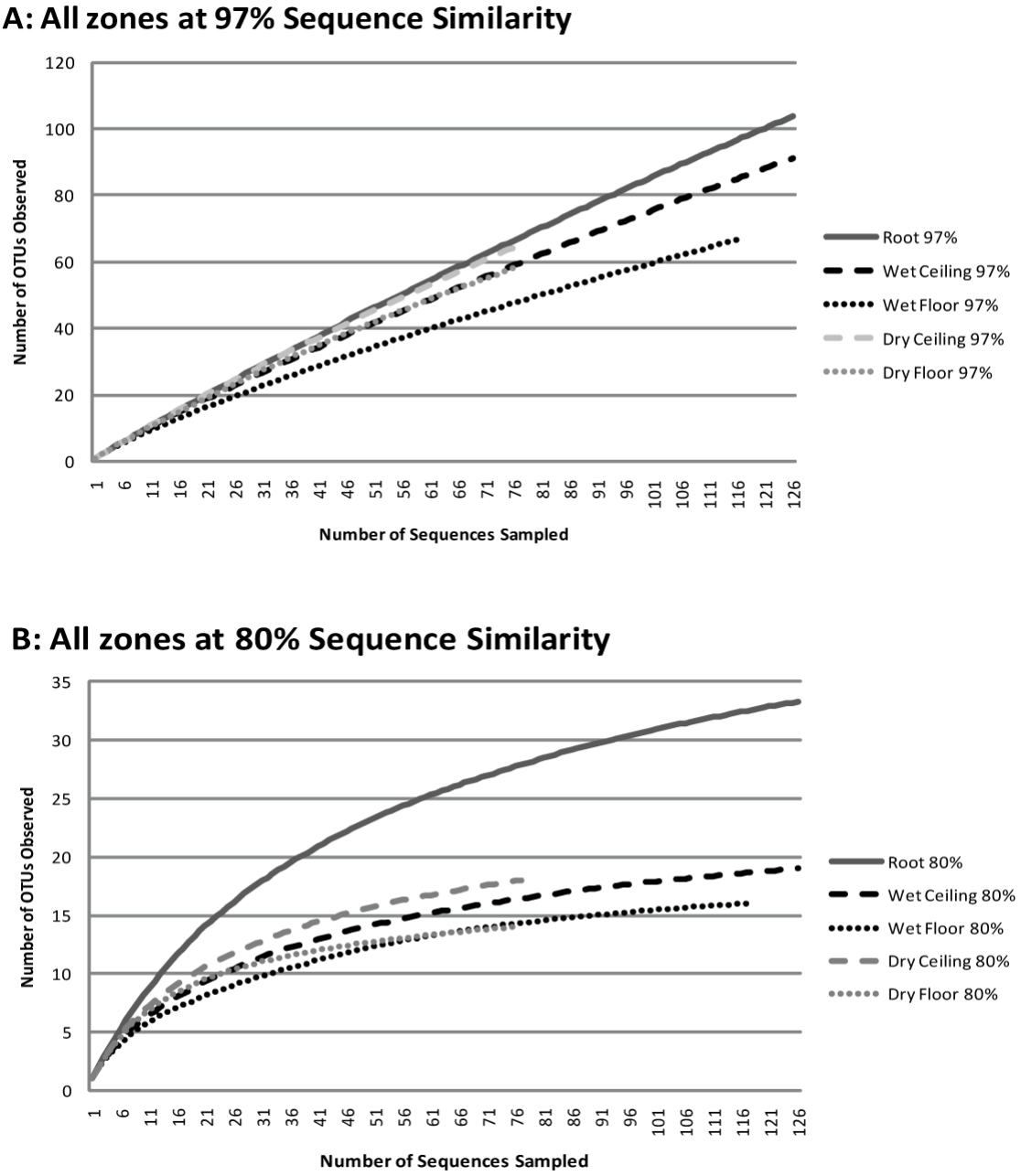


Figure 4: Rarefaction curves for all zones in Roots Galore and Pahoehoe Cave. Curves are shown at 97% sequence similarity, assumed to be at the "species" level, and the 80% sequence similarity, assumed to be at the "phylum" level.

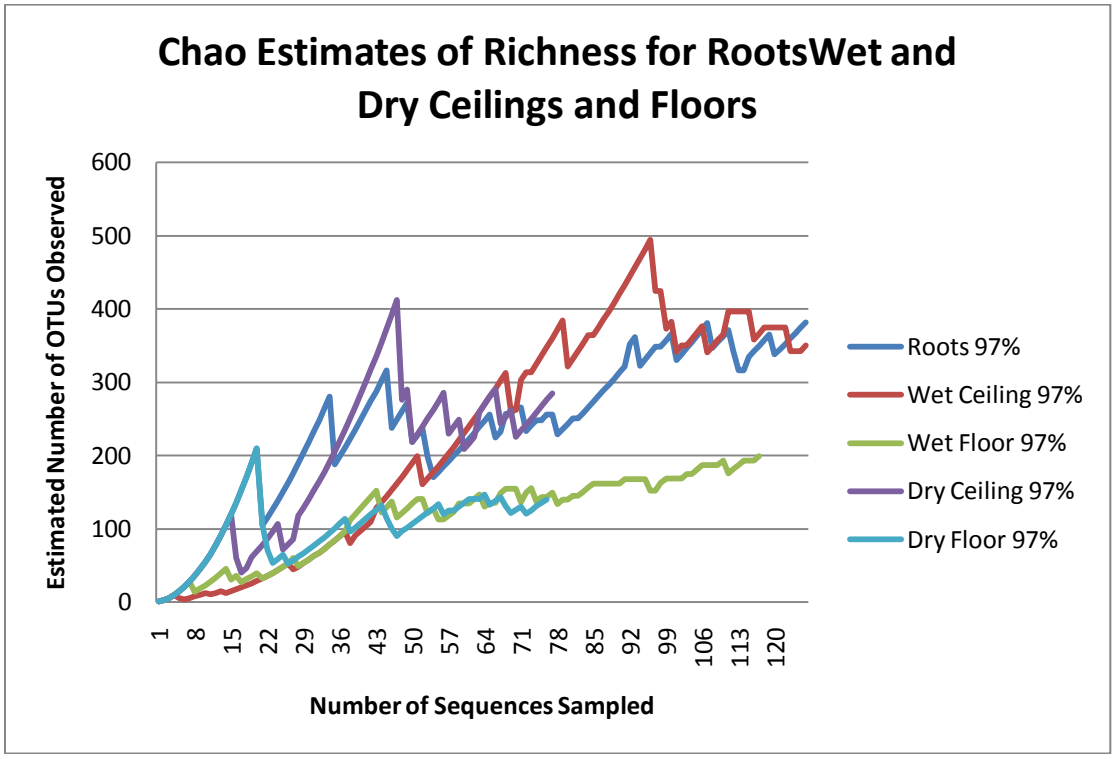


Figure 5: Chao estimates of richness of all zones in Roots Galore and Pahoehoe Caves. Chao estimate curves are shown at the 97% sequence similarity level.

Table 2: Diversity and richness of all zones in Roots Galore and Pahoehoe Caves. All calculations were completed using Dotur with the programs' standard equations.

	Roots			
	97%	95%	90%	80%
Richness (S)	119	105	73	35
Goods Coverage	16%	25%	45%	69%
Shannon-Weaver (H')	4.65 ± 0.28	4.49 ± 0.28	4.01 ± 0.30	3.17 ± 0.29
Simpsons Index (1/D)	178	125	55	21
S_{ACE}	693 ± 987.4	353.2 ± 321	131.9 ± 88.7	43.8 ± 25.1
Chao	410.2 ± 372.7	255.2 ± 199.2	116.3 ± 76.2	46 ± 41.34

	Wet Ceiling			
	97%	95%	90%	80%
Richness (S)	91	73	50	19
Goods Coverage	18%	26%	38%	79%
Shannon-Weaver (H')	4.25 ± 0.37	3.71 ± 0.4	3.34 ± 0.42	2.34 ± 0.36
Simpsons Index (1/D)	65	38	19	8
S_{ACE}	477.3 ± 635.3	302.3 ± 342.4	141.8 ± 158.6	22.2 ± 15.2
Chao	350.1 ± 399	208 ± 226.9	105.1 ± 116.2	21.5 ± 16.6

	Wet Floor			
	97%	95%	90%	80%
Richness (S)	67	61	44	16
Goods Coverage	24%	28%	48%	88%
Shannon-Weaver (H')	3.72 ± 0.47	3.56 ± 0.48	3.23 ± 0.44	2.08 ± 0.4
Simpsons Index (1/D)	23	20	16	6
S_{ACE}	314.6 ± 409.7	244.1 ± 340.3	74.8 ± 64.3	18.4 ± 13.1
Chao	199.6 ± 231.7	143.5 ± 145.7	71.6 ± 65.2	16.6 ± 7.1

	Dry Ceiling			
	97%	95%	90%	80%
Richness (S)	65	61	45	18
Goods Coverage	15%	22%	36%	72%
Shannon-Weaver (H')	4.11 ± 0.34	4.00 ± 0.35	3.60 ± 0.39	2.49 ± 0.41
Simpsons Index (1/D)	195	127	46	10
S_{ACE}	283.9 ± 395	191.5 ± 229.2	102.6 ± 107.3	21.9 ± 17
Chao	285 ± 412.4	185.3 ± 229.8	99.4 ± 119.8	20 ± 13.7

	Dry Floor			
	97%	95%	90%	80%
Richness (S)	58	51	35	14
Goods Coverage	24%	31%	46%	86%
Shannon-Weaver (H')	3.95 ± 0.37	3.75 ± 0.4	3.25 ± 0.42	2.33 ± 0.34
Simpsons Index (1/D)	98	56	25	9.4
S_{ACE}	191.5 ± 260	147.1 ± 192.2	71.9 ± 76.4	15.7 ± 11.1
Chao	140.5 ± 145.7	103.5 ± 101.2	62.1 ± 72.5	15.5 ± 14.9

Distribution of Phyla

Clones from the Root Zones grouped with closest relatives from *Acidobacteria* (26%), *Actinobacteria* (15%), *Bacteroidetes* (9%), and *Chloroflexi* (9%). *Plantomycetes*, *Nitrospirae*, *OP10*, *Gemmatimonadetes*, *Verrcomicrobia*, *Cyanobacteria*, and *TM7* were represented by less than 2% of total recovered sequences and were grouped together as *Others* (9%) (Figure 6). The Root Zone clones of the *Proteobacteria*, which represented 32% of the sequenced clones, were composed of 20% *Alphaproteobacteria*, 7% *Gammaproteobacteria*, 4% *Betaproteobacteria*, and 1% *Deltaproteobacteria*. Clones from the Wet Ceiling Zone grouped within the *Acidobacteria* (42%) *Actinobacteria* (27%), *Proteobacteria* (16%), and *Others* (13%), which included *Chloroflexi*, *Plantomycetes*, *Nitrospirae*, *Gemmatimonadetes*, *Verrcomicrobia*, *Firmicutes*, *Cyanobacteria*, *SPAM* and *TM7* (Figure 6). Dry Ceiling Zone clones grouped within *Acidobacteria* (38%), *Proteobacteria* (37%), *Bacteroidetes* (8%), *Others* (15%) and *Actinobacteria* (2%) (Figure 6). Wet Floor Zone clones grouped within *Acidobacteria* (29%), *Actinobacteria* (29%) and *Proteobacteria* (22%), *Chloroflexi* (7%), *Bacteroidetes* (3%) and *Others* (10%) while Dry Floor Zone clones included the *Proteobacteria* (52%), *Acidobacteria* (36%), *Actinobacteria* (8%), *Bacteroidetes* (8%) and *Others* (15%) (Figure 6).

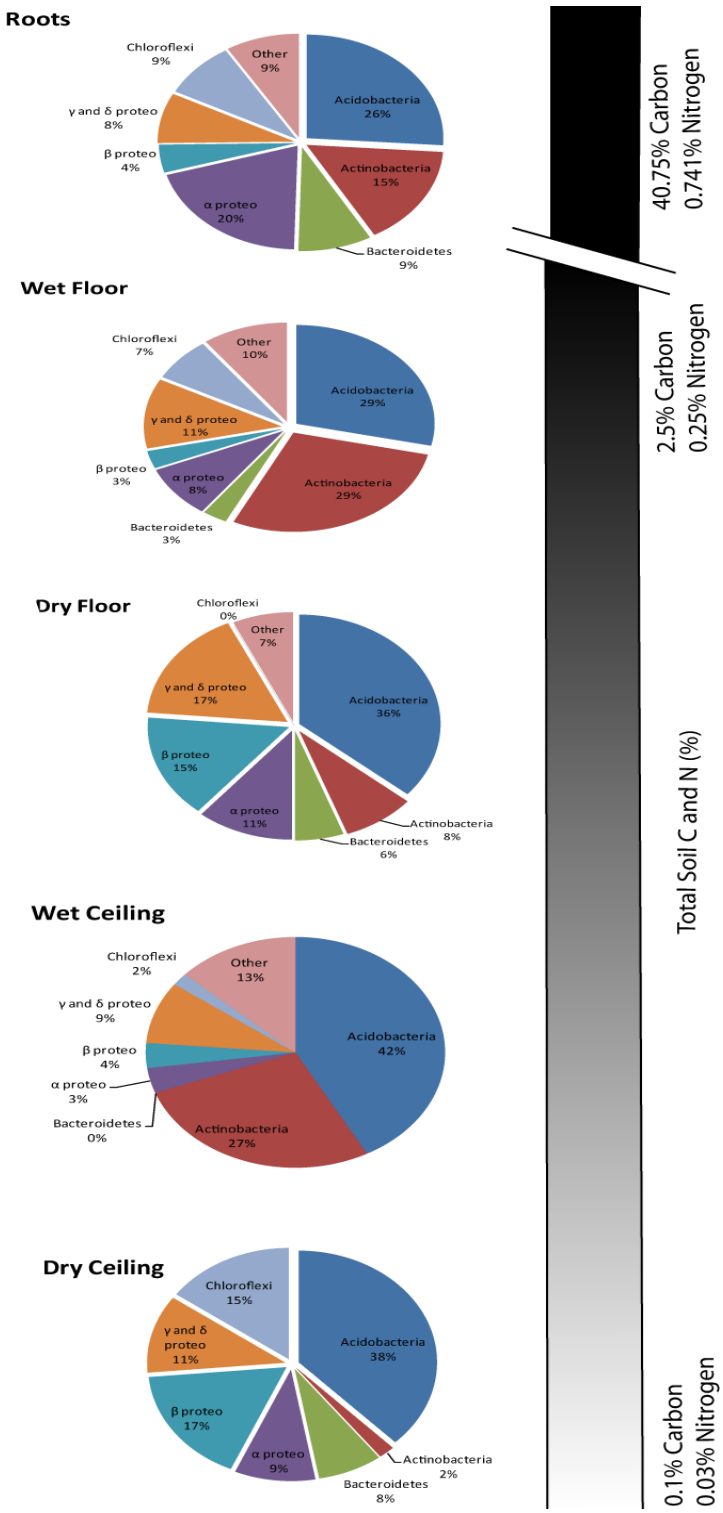


Figure 6: Pie charts of percentage of major phyla in Root, Ceilings and Floor Zones in Roots Galore and Pahoehoe Caves. The category *Others* includes *Plantomycetes, Nitrospirae*, *Gemmatimonadetes*, *Verrcomicrobia*, *Firmicutes, Cyanobacteria*, *SPAM* and *TM7* and *OP10.*

Habitats of Closest Relatives

Analysis of closest relatives of Root, Wet and Dry Ceiling and Floor Zones sequences showed that many of the nearest neighbors were uncultured relatives from other soils, rhizospheres, limestone caves, lava tube caves, simulated low waste water treatment biofilms, or other surface basaltic environments (Figure 7). Only closest relatives from soils, other cave sites and other rhizosphere sites were found at all six sampling zones. Of particular interest in this study is the percentage of clones that have cave, rhizosphere and soil related closest relatives. In the Root, Wet Ceiling, Wet Floor, Dry Ceiling and Dry Floor Zones, 14%, 13%, 25%, 12%, and 14%, respectively, of clones grouped with clones from other cave studies, 20%, 8%, 8%, 11%, and 26%, respectively, of the clones grouped with rhizsophere related isolates and 47%, 61%, 44%. 56% and 46%, respectively, of the clones grouped with bacteria that were found in soil related studies (Figure 8). The Root and Dry Ceiling Zones had significantly (Fisher Exact test) higher percentage of sequences with rhizosphere related relatives. There was no significant difference in the percentage of sequences with cave related or soil related closest relatives among the different zones (Figure 8).

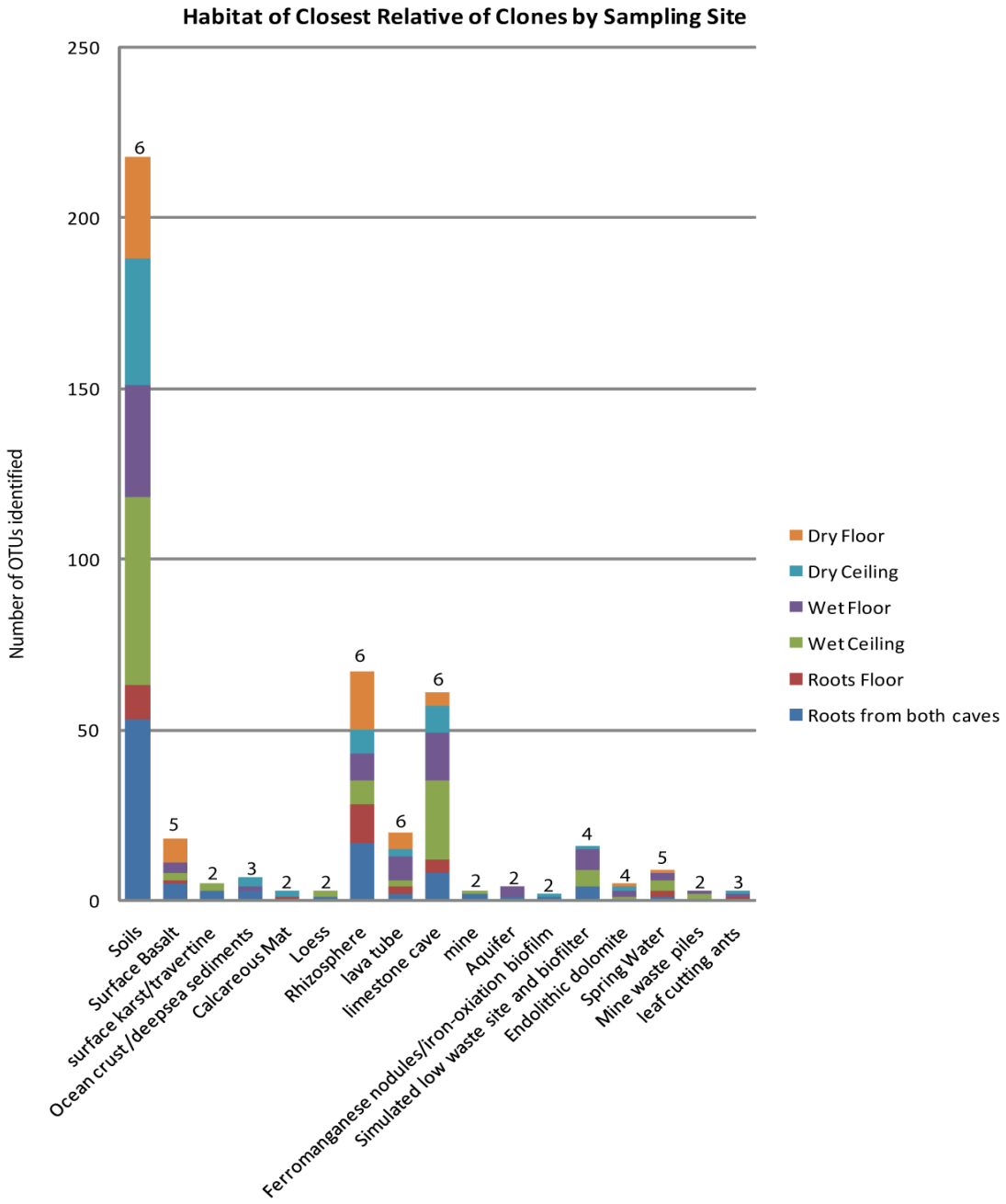


Figure 7: Habitat of closest relatives of all clones by sampling zone. Number above bar shows the number of zones in which the OTUs were found.

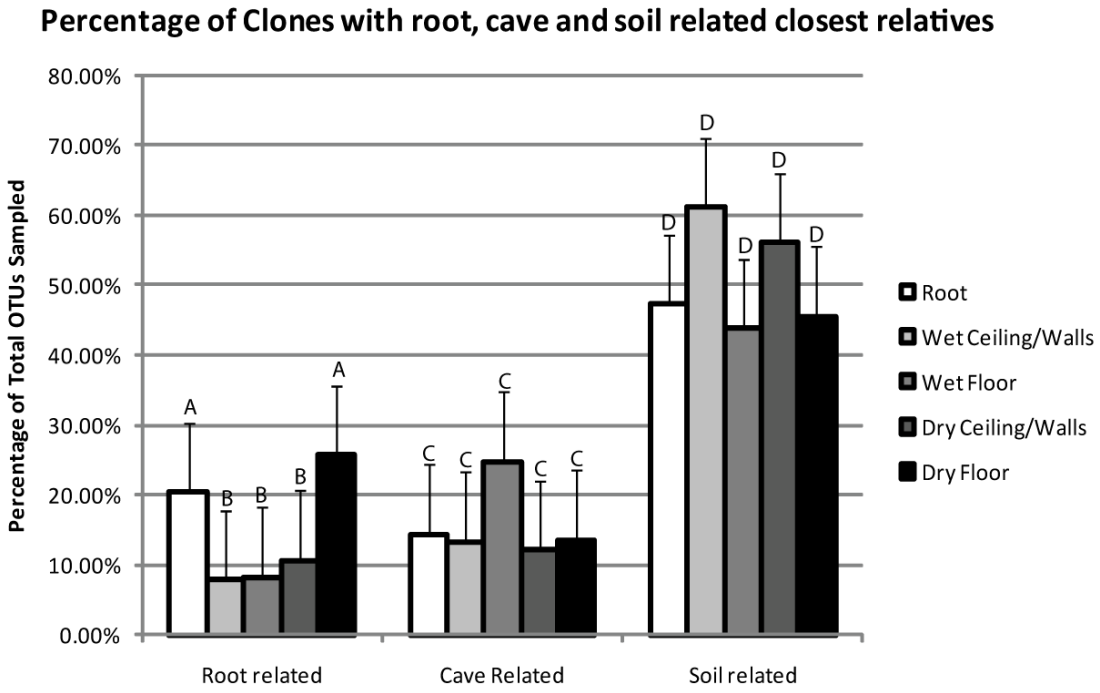


Figure 8: Percentage of clones with rhizosphere, cave and soil related closest relatives at Root and Wet and Dry Ceilings and Floors Zones in Roots Galore and Pahoehoe Caves. Letters above bars show groups that are significantly similar using Fisher's Exact Test (Minitab v15).

Microbial Community Composition

Community analysis using *mothur* and UniFrac showed that significant differences existed in microbial community composition of the Root Zones and the Wet and Dry Ceiling and Floor Zones at the 97% sequence similarity level. Venn diagrams created in *mothur* showed that only 19 OTUs or 5.12% of the total OTUs identified at the 97% sequence similarity level were shared between the Root Zone and the grouped Wet and Dry Ceiling and Floor Zones (Figure 9). Libshuff ($P<0.0001$), Parsimony ($P<0.001$), unweighted UniFrac ($P<0.001$) and weighted UniFrac ($P<0.001$) analysis showed no significant differences between the Root Zones in each of the caves, but significant

differences among Root, Wet Ceiling, Wet Floor, Dry Ceiling and Dry Floors zones. UniFrac jackknife clustering showed that the microbial communities were different from each other, with Root Zones in both caves grouping together with Roots Galore Cave's Root Floor Zone, Pahoehoe Cave's Dry Floor Zone and Roots Galore Cave's Wet Zones. Principal Coordinate Analysis (PCoA) showed that the Root Zones clustered together (cluster A in Figure 10) according to Principal Coordinate one and two, which accounted for 19.6% and 15.9% of the variation. No additional clustering was noticed corresponding to zone, but weak clustering according to Cave (Roots Galore or Pahoehoe Cave; Figure 10) was found according to Principal Coordinate one, which accounted for 19.6% of the variation (Figure 10).

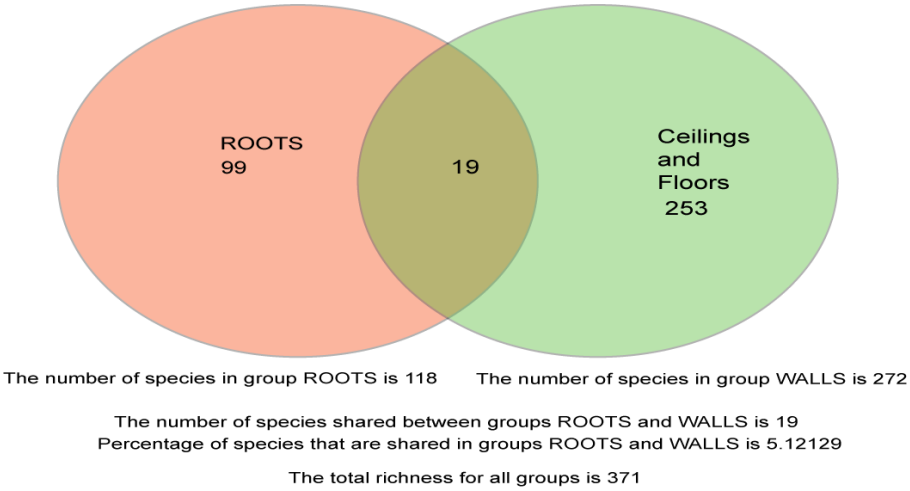


Figure 9: Venn Diagram at 97% sequence similarity level for Roots and Ceilings and Floors zones in Roots Galore and Pahoehoe Caves.

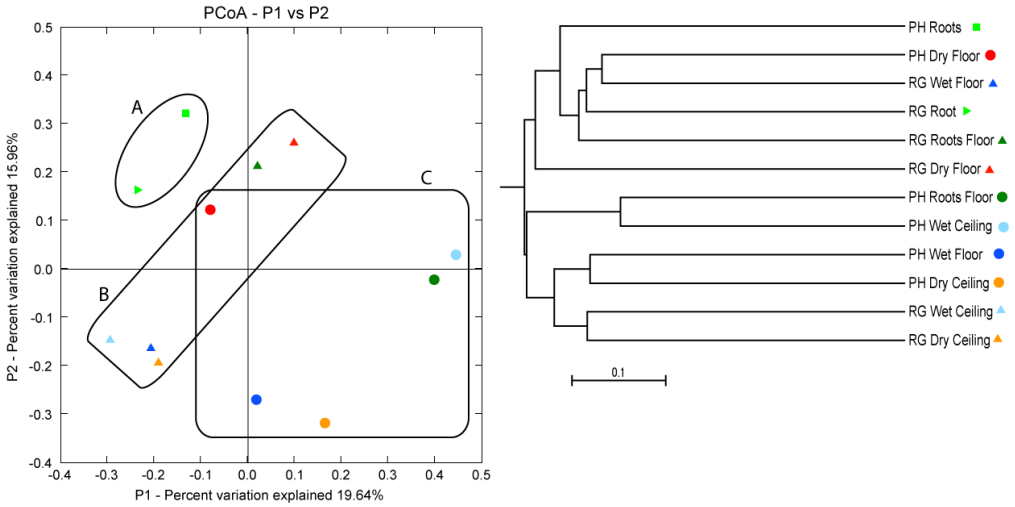


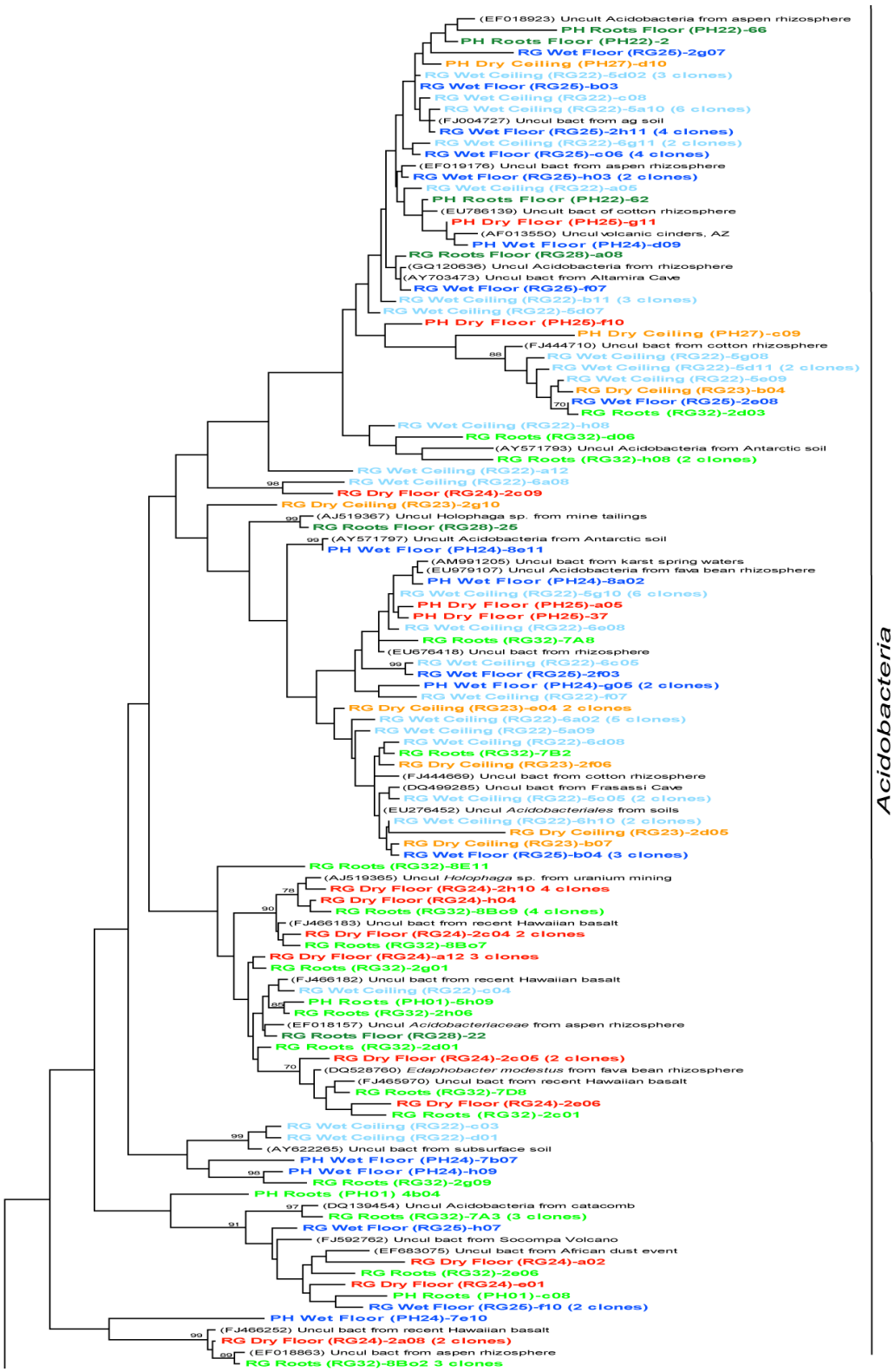
Figure 10: Principal coordinate analysis (PCoA) and jackknife cluster analysis of microbial communities in each zone. In the PCA cluster, color-coding is as follows: Light Green = Root Zones; Dark Green=Root Floor Zone; Light Blue=Wet Ceiling Zone; Dark Blue=Wet Floor Zone; Orange=Dry Ceiling Zone; and Red=Dry Floor Zone. Cluster A represents Root Zone, cluster B represents clone libraries from Pahoehoe Cave and cluster C represent clone libraries from Roots Galore Cave.

Table 3. Root associated closest relatives of RG isolates. Max identity was determined by the NCBI BLAST database query.

Location	Isolate	Closest Relative (Uc=Uncultured clone)	CR genbank #	% Max Indentity	Phylum
RG Root	RG32-a01	Uc 3H-11 from cotton Rhizosphere	EU786130	96	Plantomycetales
RG Root	RG32-b03	Uc 3y-3 from Cotton Rhizosphere	EU786138	96	Actinobacteria
RG Root	RG32-c08	Uc Elev_710 assoc. w/trembling aspen rhizosphere	EF019454	95	Bacteroidetes
RG Root	RG32-2d03	Uc 3y-44 from cotton rhizosphere	FJ444710	97	Acidobacteria
RG Root	RG32-7A1	Bradyrhizosbiaceae sp. Ai1a-2 from Costa Rica	AF514703	94	Alpha proteo
RG Root	RG32-7A8	Uc 148D1 from rhizosphere of contaminated soils	EU676418	97	Acidobacteria
RG Root	RG32-7C3	Mucilanginibacter sp DR-f2 for rhizosphere	GU139695	97	Bacteroidetes
RG Root	RG32-8B02	Uc Amb_1345 assoc. w/trembling aspen rhizosphere	EF018863	98	Acidobacteria
RG Root	RG32-2B08	Uc Elev_1214 assoc. w/trembling aspen rhizosphere	EF019856	98	Alpha proteo
RG Root	RG32-2D01	Uc Amb_492 assoc. w/trembling aspen rhizosphere	EF018157	99	Acidobacteria
RG Root	RG32-2H05	Uc Amb_1391 assoc. w/trembling aspen rhizosphere	EF018899	94	Alpha proteo
PH Root	PH01-b05	Uc Amb_583 assoc. w/trembling aspen rhizosphere	EF018232	94	Plantomycetales
PH Root	PH01-c08	Uc Elev_1240 assoc. w/trembling aspen rhizosphere	EF019878	98	Acidobacteria
PH Root	PH01-c09	Uc g72 from fava bean rhizosphere	EU979081	98	Gamma proteo
PH Root	PH01-g04	Uc Elev_493 assoc. w/trembling aspen rhizosphere	EF019302	92	Bacteroidetes
PH Root	PH01-h11	Uc Elev_493 assoc. w/trembling aspen rhizosphere	EF019302	95	Bacteroidetes
PH Root	PH01-4a02	Rhizobium sp assoc. w/prairie grasses	DQ499527	85	Bacteroidetes
PH Root	PH01-4d09	Uc SRRT57 from Phragmite rhizosphere at River in Japan	AB240487	97	Beta proteo
PH Root	PH01-4d12	Uc Elev_1427 assoc. w/trembling aspen rhizosphere	EF020032	99	Bacteroidetes
PH Root	PH01-5a12	Variovorax ginsenisoli from ginseng field	AB245358	93	Beta proteo
PH Root	PH01-5c06	Uc Amb_1313 assoc. w/trembling aspen rhizosphere	EF018832	96	Alpha proteo
RG Root Floor	RG28-b05	Uc Elev_1427 assoc. w/ trembling aspen rhizosphere	EF020032	95	Bacteroidetes
RG Root Floor	RG28-d04	Uc Amb_1192 assoc. w/ trembling aspen rhizosphere	EF018736	94	Nitrospirae
RG Root Floor	RG28-f02	Uc SRRB40 from Phragmite rhizosphere at River in Japan	AB240513	85	Chloroflexi
RG Root Floor	RG28-h03	Uc Amb_1861 assoc. w/ trembling aspen rhizosphere	EF019190	98	Plantomycetales
PH Root Floor	PH22-4	Uc Elev_493 assoc. w/trembling aspen rhizosphere	EF019302	88	Bacteroidetes
PH Root Floor	PH22-62	Uc 3y-6 from Cotton Rhizosphere	EU786139	97	Acidobacteria
PH Root Floor	PH22-70	Uc 3H-18 from Cotton Rhizosphere	EU786122	98	Gamma proteo
PH Root Floor	PH22-66	Uc Elev_1427 assoc. w/trembling aspen rhizosphere	EF020032	91	Bacteroidetes
RG Wet Ceiling	RG22-5e01	Uc Amb-717 assoc./ trembling aspen rhizosphere	EF019460	94	Nitrospirae
RG Wet Ceiling	RG22-5g06	Uc 4h-38 from Cotton Rhizosphere	FJ444732	90	Delta proteo
RG Wet Ceiling	RG22-6a08	Uc Amb_639 assoc. w/trembling aspen rhizosphere	EF018279	97	Acidobacteria
RG Wet Ceiling	RG22-6c01	Uc Elev_1324 assoc. w/trembling aspen rhizosphere	EF019945	95	Beta proteo
RG Wet Ceiling	RG22-6d08	Uc 2y-18 from Cotton Rhizosphere	FJ444669	97	Acidobacteria
RG Wet Ceiling	RG22-a05	Uc Amb_1833 assoc. w/trembling aspen rhizosphere	EF019176	96	Acidobacteria
RG Wet Ceiling	RG22-f07	Uc g73-I-25 from faba bean rhizosphere	EU979107	96	Acidobacteria
RG Wet Floor	RG25-f07	Uc P2-A6 from rhizospheres of contaminated soils	GQ120636	97	Acidobacteria
RG Wet Floor	RG25-2e01	Uc Amb_664 assoc. w/trembling aspen rhizosphere	EF018302	99	Verrucomicrobia
PH Wet Floor	PH24-b06	Pseudonocardia acaciae from Acacia roots	EU921261	95	Actinobacteria
PH Wet Floor	PH24-f04	Uc g78 from fava bean rhizosphere	EU979087	94	Alpha proteo
PH Wet Floor	PH24-g05	Uc g48 from fava bean rhizosphere	EU979057	97	Acidobacteria
PH Wet Floor	PH24-h09	Uc 69LR from rhizosphere in ag soil	FJ004768	95	Acidobacteria
PH Wet Floor	PH24-7e10	Uc Amb_844 assoc. w/trembling aspen rhizosphere	EF018576	91	Acidobacteria
PH Wet Floor	PH24-8f11	Uc 3y-19 from Cotton Rhizosphere	FJ444702	92	Delta proteo
RG Dry Ceiling	RG23-a07	Bacillus vireti from wheat rhizosphere	EU221371	96	Firmicutes
RG Dry Ceiling	RG23-g11	Uc Amb_1014 assoc. w/ trembling aspen rhizosphere	EF018401	95	Bacteroidetes
RG Dry Ceiling	RG23-2a08	Uc PR35from drought tolerant grass, Lasiurus sindicus	DQ298352	94	Actinobacteria
RG Dry Ceiling	RG23-2a09	Uc 2y-20 from Cotton Rhizosphere	FJ444671	95	Division SPAM
PH Dry Ceiling	PH27-e02	Uc g45 from fava bean rhizosphere	EU979054	99	Gemmatimonadetes
RG Dry Floor	RG24-a12	Uc Amb-1837 assoc. w/trembling aspen rhizosphere	EF019179	95	Acidobacteria
RG Dry Floor	RG24-g03	Luteibacter rhizovicinus on orchids	GU391468	97	Gamma proteo
RG Dry Floor	RG24-h07	Uc Amb_1269 assoc. w/trembling aspen rhizosphere	EF018801	97	Gamma proteo
RG Dry Floor	RG24-2c12	Uc Amb_1085 assoc. w/trembling aspen rhizosphere	EF018459	96	Alpha proteo
RG Dry Floor	RG24-2e01	Uc Amb_981 assoc. w/trembling aspen rhizosphere	EF018690	96	Alpha proteo
RG Dry Floor	RG24-2g03	Bradyrhizobium sp CTAW71 from root nodule	EU561074	99	Alpha proteo
RG Dry Floor	RG24-2g04	Bradyrhizobium sp. IV-102 from grassland roots	AB531426	97	Alpha proteo
RG Dry Floor	RG24-2h03	Variovorax soil from greenhouse soils	DQ432053	98	Beta proteo
RG Dry Floor	RG24-2h07	Uc Bradyrhizobium from truffles	DQ303345	99	Alpha proteo
PH Dry Floor	PH25-a11	Uc Amb_1014 assoc. w/trembling aspen rhizosphere	EF018401	95	Bacteroidetes
PH Dry Floor	PH25-e11	Uc Amb_416 assoc. w/trembling aspen rhizosphere	EF018086	94	Nitrospirae
PH Dry Floor	PH25-f01	Uc g63 from fava bean rhizosphere	EU979072	91	Acidobacteria
PH Dry Floor	PH25-h05	Uc g35 from fava bean rhizosphere	EU979044	92	Bacteriodetes
PH Dry Floor	PH25-g05	Uc C16.58WL fromm Lodgepole pine rhizosphere	AF432617	92	Gemmatimonadetes
PH Dry Floor	PH25-44	Uc g26 from fava bean rhizosphere	EU979035	97	Actinobacteria
PH Dry Floor	PH25-36	Uc 3y-3 from Cotton Rhizosphere	EU786138	95	Actinobacteria

Table 4. Cave associated closest relatives of Roots Galore and Pahoehoe Cave isolates. Max identity was determined by the NCBI BLAST database query.

Location	Isolate	Closest Relative (Uc=Uncultured clone)	CR genbank #	% Max Identity	Phylum
RG Root	RG32-a08	Uc 2PJM54 from Slovenia wall biofilm	FJ535083	97	Actinobacteria
RG Root	RG32-a11	Uc CV64 from Frasassi cave wall biofilm	DQ499307	98	Nitrospira
RG Root	RG32-h12	Uc RSC-LGE10 from Orthoquartize cave	GU205310	97	Actinobacteria
RG Root	RG32-2a09	Uc RSC-LGE10 from Orthoquartize cave	GU205310	94	Actinobacteria
RG Root	RG32-2h12	Uc CV22 from wall biofilms from Frasassi cave	DQ499282	96	Nitrospira
RG Root	RG32-7A3	Uc Cal2 from biofilm in Roman catacombs	DQ139454	97	Acidobacteria
RG Root	RG32-8C11	Uc HAVOmat18 from Hawaiian lava cave	EF032763	95	Bacteroidetes
RG Root	RG32-8G09	Uc RSC-LGE10 from Orthoquartize cave	GU205310	97	Actinobacteria
PH Root	PH01-4d01	Uc HAVOmat81 from Hawaiian lava cave	EF032763	91	Bacteroidetes
PH Root	PH01-4e08	Cult. Alpha proteobacteria from Kartchner Caverns, AZ	FJ11221	92	Alpha proteo
PH Root	PH01-5a02	Uc CV22 from wall biofilms from Frasassi Cave	DQ499282	97	Nitrospira
PH Root	PH01-5h11	Uc C05-2 from biofilm in gold mine	FM253645	97	Chloroflexi
RG Root Floor	RG28a08	Uc Alt2 from Altamira Cave	AY703473	98	Acidobacteria
RG Root Floor	RG28e04	Uc Foos8B073 from troglobitic beetles	EU431813	92	Delta proteo
RG Root Floor	RG28-21	Uc RSC-LGE10 from Orthoquartize cave	GU205310	97	Actinobacteria
PH Root Floor	PH22-46	Uc ORCA-17F09 from Oregon Caves National Monument	DQ823191	88	Gammproteo
PH Root Floor	PH22-47	Uc ORCA-17N105 from Oregon Caves National Monument	DQ823212	94	Gamma proteo
RG Wet Ceiling	RG22-2	Uc CV26 from wall biofilms from Frasassi Cave	DQ499285	97	Acidobacteria
RG Wet Ceiling	RG22-5e10	Uc 2PJM17 from Slovenia cave wall biofilm	FJ525097	95	Nitrospira
RG Wet Ceiling	RG22-5e11	Uc CV22 from wall biofilms from Frasassi cave	DQ499282	94	Nitrospira
RG Wet Ceiling	RG22-5f03	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	95	Gamma-proteo
RG Wet Ceiling	RG22-5g05	Uc H06-1 Biofilm from gold mine rock	FM253628	90	Alpha proteo
RG Wet Ceiling	RG22-6b12	Uc CV22 from wall biofilms from Frasassi cave	DQ499282	93	Nitrospira
RG Wet Ceiling	RG22-6d12	Uc MACA-CC36 from Mammoth Cave Aquifers	GQ500727	97	Alpha proteo
RG Wet Ceiling	RG22-6h01	Uc ORCA-17N121 from Oregon Caves National Monument	DQ823244	98	Gamma-proteo
RG Wet Ceiling	RG22-d01	Uc S-B2 from uranium-contaminated aquifer	AY622265	97	Acidobacteria
RG Wet Ceiling	RG22-f12	Uc M6A-217 from karst groundwater	AM991205	97	Acidobacteria
PH Wet Ceiling	PH26-4	Uc 2PJM54 from Slovenia wall biofilm	FJ535083	98	Actinobacteria
PH Wet Ceiling	PH26-26	Uc RSC-LGE10 from Orthoquartize cave	GU205310	97	Actinobacteria
RG Wet Floor	RG25-a07	Uc 3PJM80 from Slovenia wall biofilm	FJ535092	98	Actinobacteria
RG Wet Floor	RG25-b06	Uc 1174-901-7 from deep subsurface sediments	AB128884	92	Delta proteo
RG Wet Floor	RG25-b09	Uc 2PJM54 from Slovenia wall biofilm	FJ535083	98	Actinobacteria
RG Wet Floor	RG25-d07	Uc 2PJM54 from Slovenia wall biofilm	FJ535083	97	Actinobacteria
RG Wet Floor	RG25-d08	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	97	Gamma-proteo
RG Wet Floor	RG25-d09	Uc HAVOmat81 from Hawaiian lava cave	EF032765	93	Bacteroidetes
RG Wet Floor	RG25-e04	Uc MACA-EFT48 from Mammoth Cave Aquifers	GQ500812	98	Alpha proteo
RG Wet Floor	RG25-g03	Uc MACA-RR16 from Mammoth Cave Aquifers	GQ500751	97	Nitrospira
RG Wet Floor	RG25-g12	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	94	Gamma-proteo
RG Wet Floor	RG25-2b08	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	95	Gamma-proteo
PH Wet Floor	PH24-c04	Uc CV54 from wall biofilms from Frasassi cave	DQ499303	97	Unclassifed/Actino
PH Wet Floor	PH24-c10	Uc MACA-RR33 from Mammoth Cave Aquifers	GQ500765	99	Nitrospira
RG Dry Ceiling	RG23-c08	Uc 1PJM279 from Slovenia cave wall biofilm	FJ535096	93	Chloroflexi
RG Dry Ceiling	RG23-e05	Uc 5B-8 from subsurface soil in Oman	DQ906811	94	Candidate SPAM
RG Dry Ceiling	RG23-h05	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	99	Gamma-proteo
RG Dry Ceiling	RG23-2a03	Uc Z53M66B from world's deepest phreatic sinkhole	FJ484539	89	Chloroflexi
PH Dry Ceiling	PH27-b07	Uc ORCA-17N112 from Oregon Caves National Monument	DQ823216	99	Gamma proteo
PH Dry Ceiling	PH27-e03	Uc MACA-EFT48 from Mammoth Cave Aquifers	GQ500812	98	Alpha proteo
PH Dry Ceiling	PH27-h04	Uc Fe-D22 from arsenic-contaminated aquifer	EU431813	97	Actinobacteria
PH Dry Ceiling	PH27-h05	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	91	Gamma-proteo
RG Dry Floor	RG24-2h05	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	92	Gamma proteo
RG Dry Floor	PH25-b05	Uc ORCA-17F02 from Oregon Caves National Monument	DQ823185	90	Alpha proteo
RG Dry Floor	PH25-d05	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	95	Gamma proteo
RG Dry Floor	PH25-d10	Uc MACA-CC38 from Mammoth Cave Aquifers	GQ500729	91	Delta proteo
RG Dry Floor	PH25-e10	Uc MACA-CC38 from Mammoth Cave Aquifers	GQ500726	99	Gamma proteo
RG Dry Floor	PH25-f07	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	89	Gamma proteo
RG Dry Floor	PH25-g08	Uc 2PJM54 from Slovenia wall biofilm	FJ535083	98	Actinobacteria
RG Dry Floor	PH25-h10	Uc ORCA-17N118 from Oregon Caves National Monument	DQ823220	92	Gamma-proteo
RG Dry Floor	PH25-12	Uc 2PJM54 from Slovenia wall biofilm	FJ355083	100	Actinobacteria


(EF018923) Uncult Acidobacteria from aspen rhizosphere
PH Roots Floor (PH22)-66
PH Roots Floor (PH22)-2
RG Wet Floor (RG25)-2g07
PH Dry Ceiling (PH27)-d10
RG Wet Ceiling (RG22)-5d02 (3 clones)
RG Wet Floor (RG25)-b03
RG Wet Ceiling (RG22)-c08
RG Wet Ceiling (RG22)-5a10 (6 clones)
(FJ004727) Uncul bact from ag soil
RG Wet Floor (RG25)-2h11 (4 clones)
RG Wet Ceiling (RG22)-6g11 (2 clones)
RG Wet Floor (RG25)-c06 (4 clones)
(EF019176) Uncul bact from aspen rhizosphere
RG Wet Floor (RG25)-h03 (2 clones)
RG Wet Ceiling (RG22)-a06
PH Roots Floor (PH22)-62
(EU786139) Uncult bact of cotton rhizosphere
PH Dry Floor (PH25)-g11
(AF013550) Uncul volcanic cinders, AZ
PH Wet Floor (PH24)-d09
RG Roots Floor (RG28)-a08
(GQ120636) Uncul Acidobacteria from rhizosphere
(AY703473) Uncul bact from Altamira Cave
RG Wet Floor (RG25)-f07
RG Wet Ceiling (RG22)-b11 (3 clones)
RG Wet Ceiling (RG22)-5d07
PH Dry Floor (PH25)-f10
PH Dry Ceiling (PH27)-c09
(FJ444710) Uncul bact from cotton rhizosphere
RG Wet Ceiling (RG22)-6g08
RG Wet Ceiling (RG22)-5d11 (2 clones)
RG Wet Ceiling (RG22)-6e09
RG Dry Ceiling (RG23)-b04
RG Wet Floor (RG25)-2e08
RG Roots (RG32)-2d03
RG Wet Ceiling (RG22)-h08
RG Roots (RG32)-d06
(AY571793) Uncul Acidobacteria from Antarctic soil
RG Roots (RG32)-h08 (2 clones)
RG Wet Ceiling (RG22)-a12
RG Wet Ceiling (RG22)-6a08
RG Dry Floor (RG24)-2c09
RG Dry Ceiling (RG23)-2g10
(AJ519367) Uncul Holophaga sp. from mine tailings
RG Roots Floor (RG28)-25
(AY571797) Uncult Acidobbacteria from Antarctic soil
PH Wet Floor (PH24)-8e11
(AM991205) Uncul bact from karst spring waters
(EU979107) Uncul Acidobacteria from fava bean rhizosphere
PH Wet Floor (PH24)-8a02
RG Wet Ceiling (RG22)-5g10 (6 clones)
PH Dry Floor (PH25)-a05
PH Dry Floor (PH25)-37
RG Wet Ceiling (RG22)-6e08
RG Roots (RG32)-7A8
(EU676418) Uncul bact from rhizosphere
RG Wet Ceiling (RG22)-6c05
RG Wet Floor (RG25)-2f03
PH Wet Floor (PH24)-g05 (2 clones)
RG Wet Ceiling (RG22)-f07
RG Dry Ceiling (RG23)-e04 2 clones
RG Wet Ceiling (RG22)-6a02 (5 clones)
RG Wet Ceiling (RG22)-5a09
RG Wet Ceiling (RG22)-6d08
RG Roots (RG32)-7B2
RG Dry Ceiling (RG23)-2f06
(FJ444669) Uncul bact from cotton rhizosphere
(DQ499285) Uncul bact from Frasassi Cave
RG Wet Ceiling (RG22)-5c05 (2 clones)
(EU276452) Uncul Acidobacteriales from soils
RG Wet Ceiling (RG22)-6h10 (2 clones)
RG Dry Ceiling (RG23)-2d05
RG Dry Ceiling (RG23)-b07
RG Wet Floor (RG25)-b04 (3 clones)
RG Roots (RG32)-8E11
(AJ519365) Uncul Holophaga sp. from uranium mining
RG Dry Floor (RG24)-2h10 4 clones
RG Dry Floor (RG24)-h04
RG Roots (RG32)-8Bo9 (4 clones)
(FJ466183) Uncul bact from recent Hawaiian basalt
RG Dry Floor (RG24)-2c04 2 clones
RG Roots (RG32)-8Bo7
RG Dry Floor (RG24)-a12 3 clones
RG Roots (RG32)-2g01
(FJ466182) Uncul bact from recent Hawaiian basalt
RG Wet Ceiling (RG22)-c04
PH Roots (PH01)-5h09
RG Roots (RG32)-2h06
(EF018157) Uncul Acidobacteriaceae from aspen rhizosphere
RG Roots Floor (RG28)-22
RG Roots (RG32)-2d01
RG Dry Floor (RG24)-2c05 (2 clones)
(DQ528760) Edaphobacter modestus from fava bean rhizosphere
(FJ465970) Uncul bact from recent Hawaiian basalt
RG Roots (RG32)-7D8
RG Dry Floor (RG24)-2e06
RG Roots (RG32)-2c01
RG Wet Ceiling (RG22)-c03
RG Wet Ceiling (RG22)-d01
(AY622265) Uncul bact from subsurface soil
PH Wet Floor (PH24)-7b07
PH Wet Floor (PH24)-h09
RG Roots (RG32)-2g09
PH Roots (PH01) 4b04
(DQ139454) Uncul Acidobacteria from catacomb
RG Roots (RG32)-7A3 (3 clones)
RG Wet Floor (RG25)-h07
(FJ592762) Uncul bact from Socompa Volcano
(EF683075) Uncul bact from African dust event
RG Dry Floor (RG24)-a02
RG Roots (RG32)-2e06
RG Dry Floor (RG24)-e01
PH Roots (PH01)-c08
RG Wet Floor (RG25)-f10 (2 clones)
PH Wet Floor (PH24)-7e10
(FJ466252) Uncul bact from recent Hawaiian basalt
RG Dry Floor (RG24)-2a08 (2 clones)
(EF018863) Uncul bact from aspen rhizosphere
RG Roots (RG32)-8Bo2 3 clones
Acidobacteria

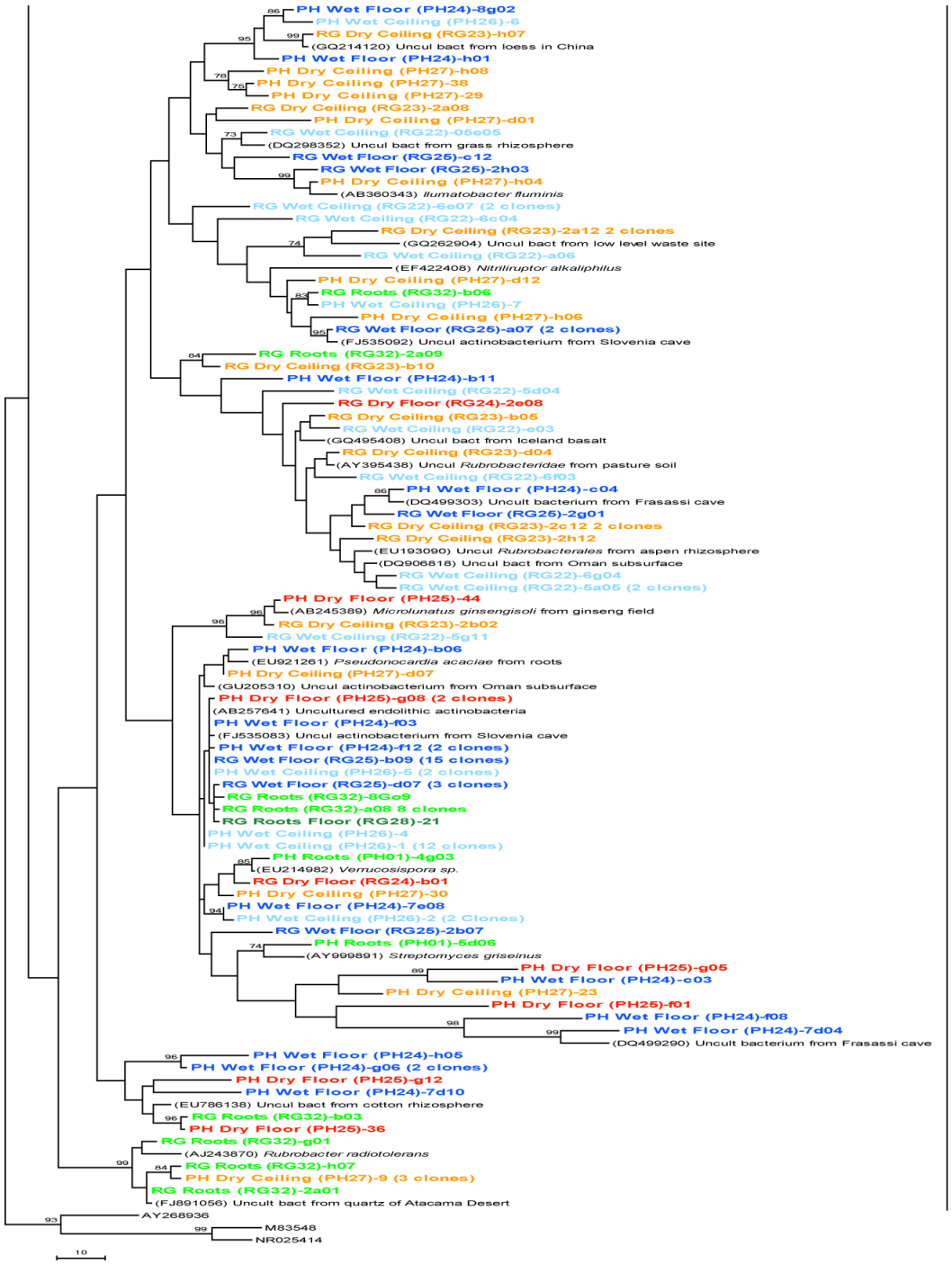
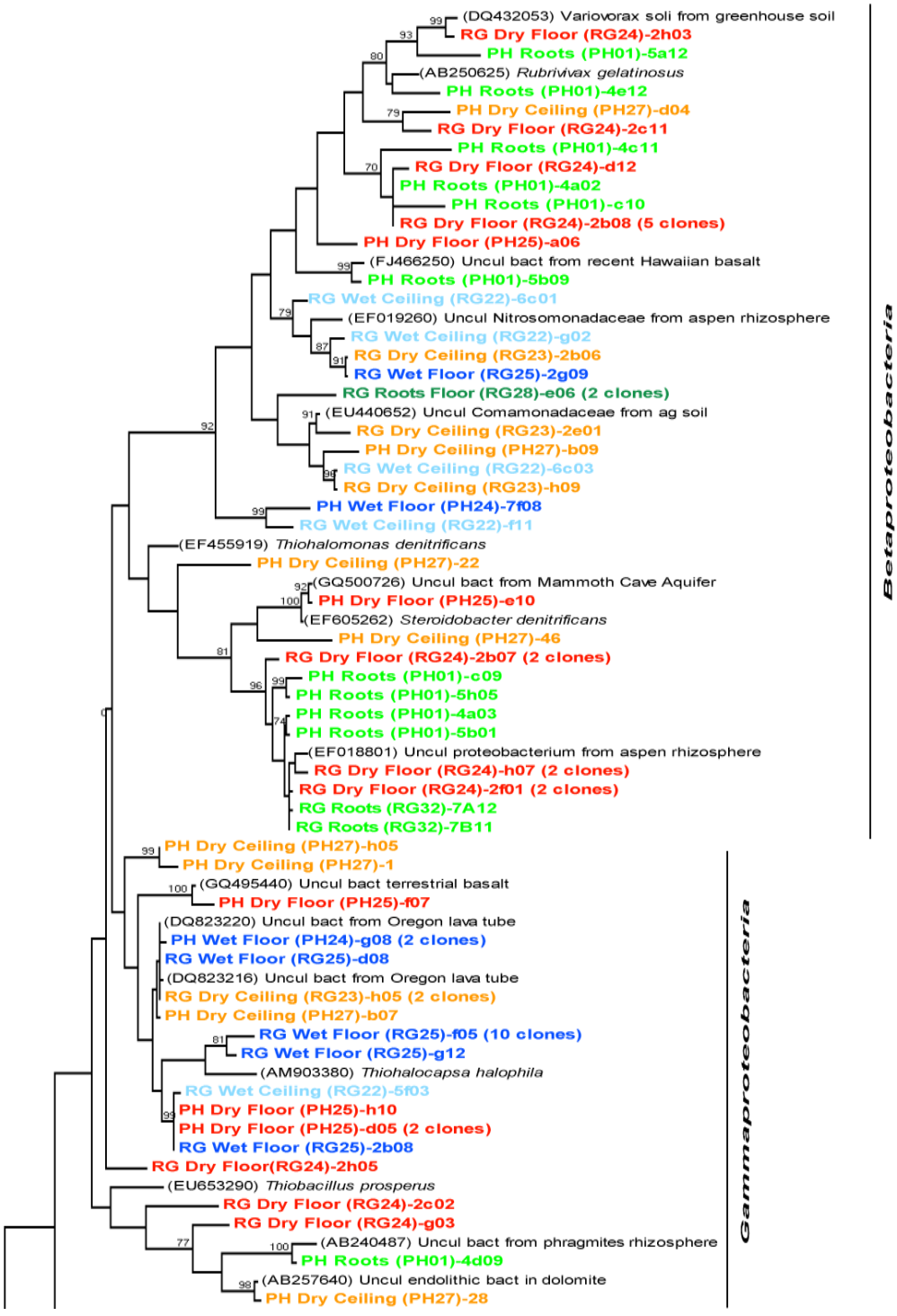


Figure 11: Maximum parsimony tree of *Acidobacteria* and *Actinobacteria* sequences found in two caves in New Mexico**.** The evolutionary history was inferred using the Maximum Parsimony method and the most parsimonious tree is shown. Bootstrap values are based on 1000 replicates and bootstrap values above 70% are shown. Clones from this study are in bold and using the following color-coding: Light Green = Root Zones; Dark Green=Root Floor Zone; Light Blue=Wet Ceiling Zone; Dark Blue=Wet Floor Zone; Orange=Dry Ceiling Zone; and Red=Dry Floor Zone.


(DQ432053) Variovorax soli from greenhouse soil
RG Dry Floor (RG24)-2h03
PH Roots (PH01)-5a12
(AB250625) Rubrivivax gelatinosus
PH Roots (PH01)-4e12
PH Dry Ceiling (PH27)-d04
RG Dry Floor (RG24)-2c11
PH Roots (PH01)-4c11
RG Dry Floor (RG24)-d12
PH Roots (PH01)-4a02
PH Roots (PH01)-c10
RG Dry Floor (RG24)-2b08 (5 clones)
PH Dry Floor (PH25)-a06
(FJ466250) Uncul bact from recent Hawaiian basalt
PH Roots (PH01)-5b09
RG Wet Ceiling (RG22)-6c01
(EF019260) Uncul Nitrosomonadaceae from aspen rhizosphere
RG Wet Ceiling (RG22)-g02
RG Dry Ceiling (RG23)-2b06
RG Wet Floor (RG25)-2g09
RG Roots Floor (RG28)-e06 (2 clones)
(EU440652) Uncul Comamonadaceae from ag soil
RG Dry Ceiling (RG23)-2e01
PH Dry Ceiling (PH27)-b09
RG Wet Ceiling (RG22)-6c03
RG Dry Ceiling (RG23)-h09
PH Wet Floor (PH24)-7f08
RG Wet Ceiling (RG22)-f11
(EF455919) Thiohalomonas denitrificans
PH Dry Ceiling (PH27)-22
(GQ500726) Uncul bact from Mammoth Cave Aquifer
PH Dry Floor (PH25)-e10
(EF605262) Steroidobacter denitrificans
PH Dry Ceiling (PH27)-46
RG Dry Floor (RG24)-2b07 (2 clones)
PH Roots (PH01)-c09
PH Roots (PH01)-5h05
PH Roots (PH01)-4a03
PH Roots (PH01)-5b01
(EF018801) Uncul proteobacterium from aspen rhizosphere
RG Dry Floor (RG24)-h07 (2 clones)
RG Dry Floor (RG24)-2f01 (2 clones)
RG Roots (RG32)-7A12
RG Roots (RG32)-7B11
PH Dry Ceiling (PH27)-h05
PH Dry Ceiling (PH27)-1
(GQ495440) Uncul bact terrestrial basalt
PH Dry Floor (PH25)-f07
(DQ823220) Uncul bact from Oregon lava tube
PH Wet Floor (PH24)-g08 (2 clones)
RG Wet Floor (RG25)-d08
(DQ823216) Uncul bact from Oregon lava tube
RG Dry Ceiling (RG23)-h05 (2 clones)
PH Dry Ceiling (PH27)-b07
RG Wet Floor (RG25)-f05 (10 clones)
RG Wet Floor (RG25)-g12
(AM903380) Thiohalocapsa halophila
RG Wet Ceiling (RG22)-5f03
PH Dry Floor (PH25)-h10
PH Dry Floor (PH25)-d05 (2 clones)
RG Wet Floor (RG25)-2b08
RG Dry Floor(RG24)-2h05
(EU653290) Thiobacillus prosperus
RG Dry Floor (RG24)-2c02
RG Dry Floor (RG24)-g03
(AB240487) Uncul bact from phragmites rhizosphere
PH Roots (PH01)-4d09
(AB257640) Uncul endolithic bact in dolomite
PH Dry Ceiling (PH27)-28
Betaproteobacteria
Gammaproteobacteria

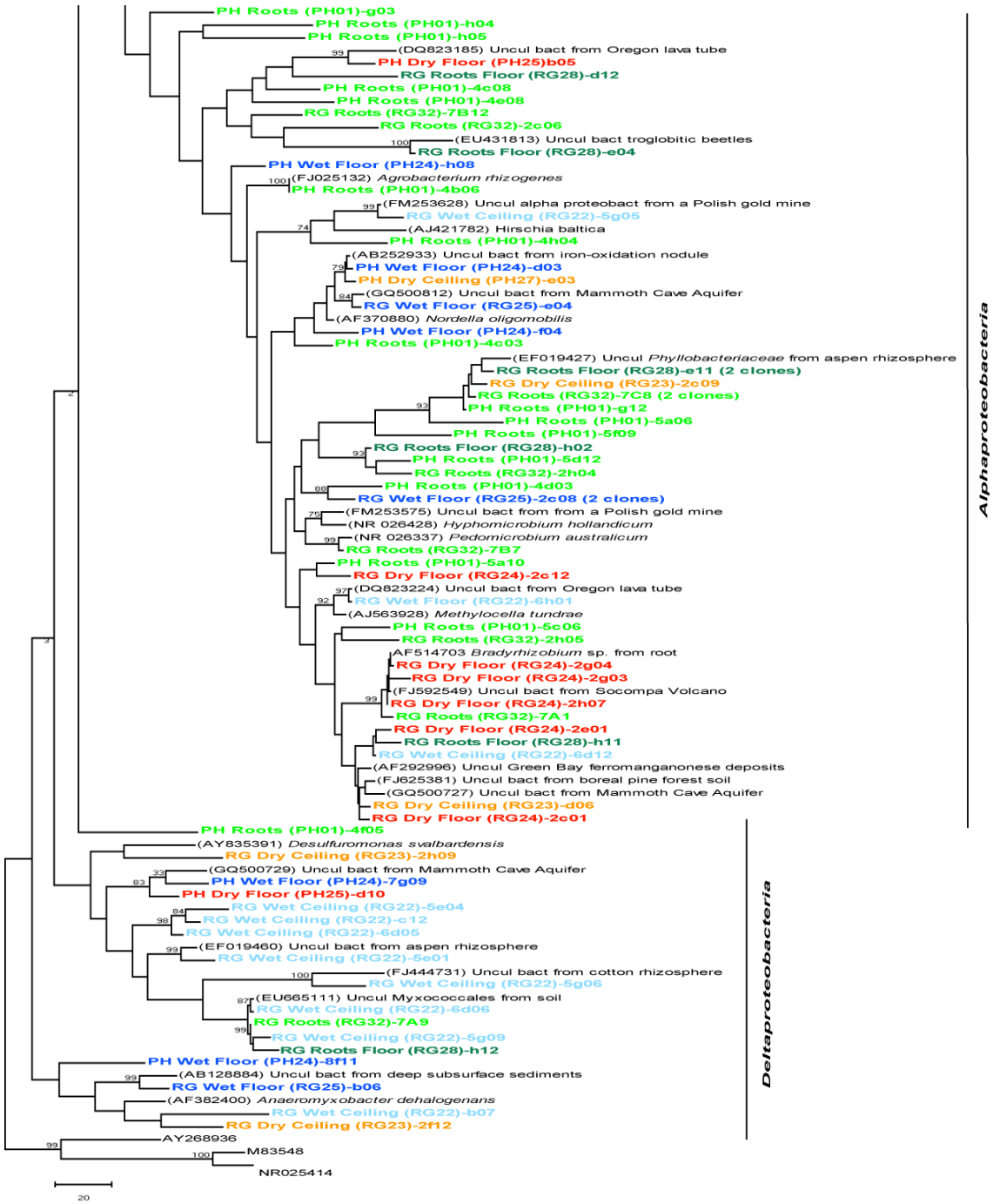


Figure 12: Maximum parsimony tree of the *Protoebacteria* sequences found in two caves in New Mexico. The evolutionary history was inferred using the Maximum Parsimony method and the most parsimonious tree is shown. Bootstrap values are based on 1000 replicates and bootstrap values above 70% are shown. Clones from this study are in bold and using the following color-coding: Light Green = Root Zones; Dark Green=Root Floor Zone; Light Blue=Wet Ceiling Zone; Dark Blue=Wet Floor Zone; Orange=Dry Ceiling Zone; and Red=Dry Floor Zone.

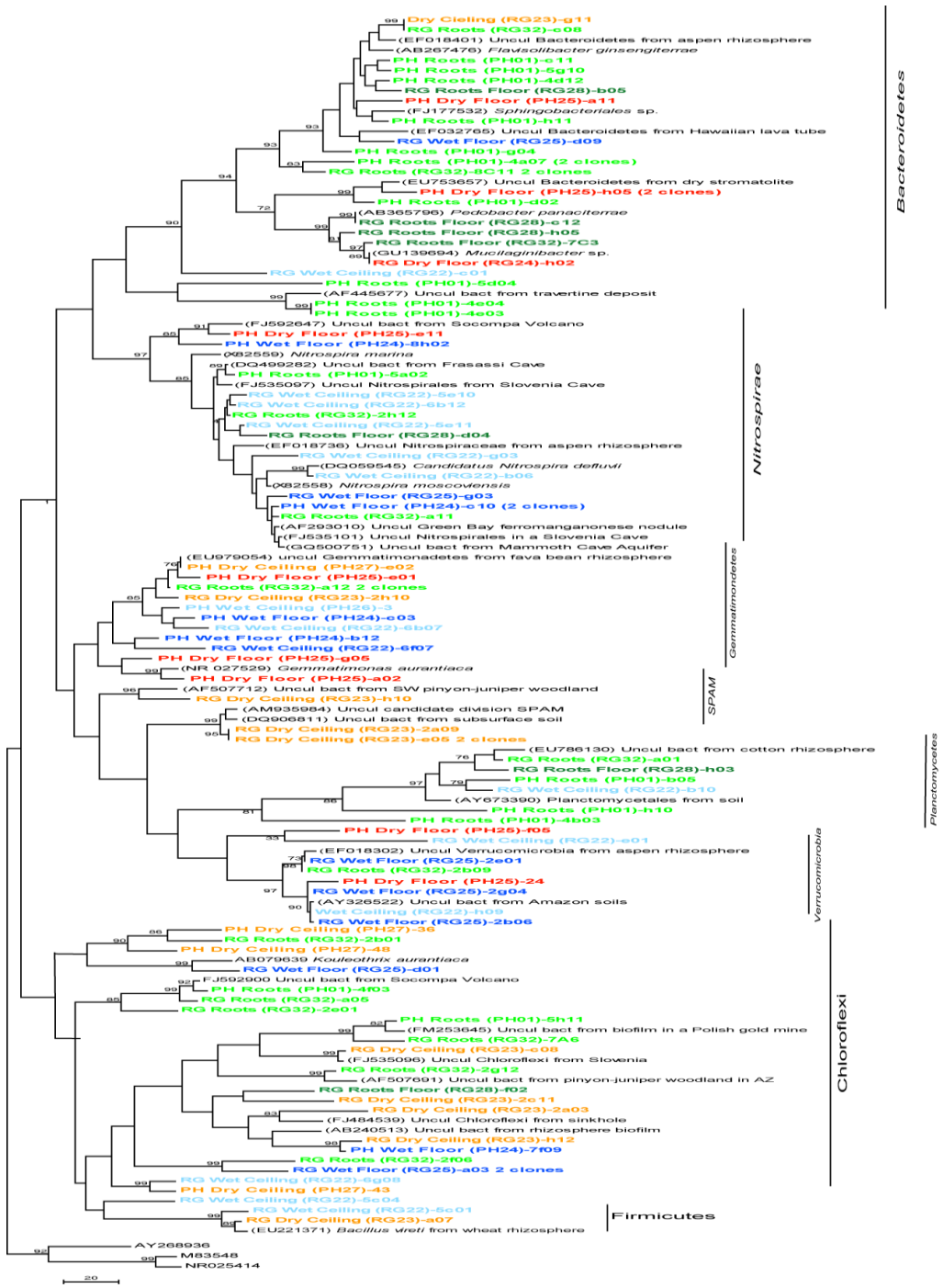


Figure 13: Maximum parsimony tree of sequences from the remaining eight phyla found in two caves in New Mexico. The evolutionary history was inferred using the Maximum Parsimony method and the most parsimonious tree is shown. Bootstrap values are based on 1000 replicates and bootstrap values above 70% are shown. Clones from this study are in bold and using the following color-coding: Light Green = Root Zones; Dark Green=Root Floor Zone; Light Blue=Wet Ceiling Zone; Dark Blue=Wet Floor Zone; Orange=Dry Ceiling Zone; and Red=Dry Floor Zone.

Table 5: Cultured relatives of clones and known metabolic pathway.

Cultured Bacteria	Metabolic Pathway	Closest Clone	Study
Streptomyces griseus	chemoorganotrophs	PH01-5d03	Kämpfer, 2006
Nitriliruptor alkaliphilus	chemoorganotrophs	PH27-d12	Sorokin et al., 2009
Ilumatobacter fluminus	chemoorganotrophs	PH27-h04	Matsumoto et al., 2009
Rubrobacter radiotolerans	chemoorganotrophs	RG32-b03	Matsumoto et al., 2009
Edaphobacter modestus	chemoorganotrophs	RG24-2c05	Koch et al., 2008
Rubrivivas gelatinosus	chemoorganotrophs	PH01-4e12	Stennon et al., 2004
Gemmatimonas aurantiaca	chemoorganotrophs	PH25-a02	Zhang et al., 2003
Bacillus vireti	chemoorganotrophs	RG23-a07	Heyrman et al., 2004
Flavisolibacter ginsengiterrae	chemoorganotrophs	RG32-c08	Yoon et al., 2007
Pedobacter panaciterrae	chemoorganotrophs	RG28-c12	Yoon et al., 2007a
Agrobacterium rhizogenes	chemoorganotrophs	PH01-4b06	Giri et al., 2001
Pseudonocardi acaciae	chemoorganotrophs	PH24-b06	Duangmal et al., 2009
Microlunatus ginsengisoli	chemoorganotrophs	PH25-44	Cui et al., 2009
Steroidobacter denitrificans	denitrifier	PH25-e10	Fahrbach et al., 2008
Hyphomicrobium hollandicum	Manganese and iron oxidizer	RG32-7B7	McDonald et al., 2001
Pedomicrobium australicum	Manganese and iron oxidizer	RG32-7B7	Gebers and Beese, 1988
Variovorax soli	Manganese oxidizer	RG24-2h03	Kim et al., 2006
Hyphomicrobium hollandicum	Methanotrophs	RG32-7B7	McDonald et al., 2001
Methylocella tundra	Methanotrophs	RG22-6h01	Dedysh et al., 2004
Nitrospira marina	Nitrite oxidizer	PH01-6a02	Daims et al., 2001
Nitrospira moscoviensis	Nitrite oxidizer	RG22-b06	Ehrich et al., 1995
Desulfuromonas svalbardensis	Sulfur and iron reducer	RG23-2h09	Vandiekan et al., 2006
Thiohalocapsa halophila	Sulfur oxidizers	RG25-f05	Sorokin et al., 2007
Thiohalomonas denitrifican	Sulfur oxidizers	PH27-22	Davis-Belmar et al. 2007
Thiobacillus prosperus	Sulfur oxidizers	PH27-h05	Davis-Belmar et al. 2007

Table 6: Percentage of clones that grouped within each phylum in the Root Zone of this study and trembling aspen rhizosphere. Data for trembling aspen derived from Lesaulnier et al., 2008

Phylum	Root Zone	Trembling aspen
Acidobacteria	26	30
Actinobacteria	15.7	7.2
Bacteroidetes	8.7	3.6
Alphaproteobacteria	20	20
Betaproteobacteria	4.3	10
Gammaproteobacteria	7	3.3
Deltaproteobacteria	0.8	3.2
Planctomycetes	1.7	2.7
Cyanobacteria	1.7	0.3
Chloroflexi	7	0.5
Nitrospirae	2.6	1
OP10	0.8	0
Gemmatimonadetes	1.7	2
Verrucomicrobia	0.9	6.2
Firmicutes	0	6.9
TM7	0.9	0.2
OD1	0	0.7
Thermomicrobia	0	0.3
Dehalococcoides	0	0.3

Table 7: Percentage of clones that grouped within each phylum in the grouped Ceiling and Floor Zones of this study, white colonies from Altamira Cave, Spain (derived from Portillo and Gonzalez, 2009) and Pajsarjeva jama Cave, Slovenia (derived from Pašić et al., 2009

Phylum	Walls and Floors	Altamira White	Pajsarjeva jama
Acidobacteria	34	21.4	17
Actinobacteria	20	0	28
Bacteriodetes	3	0	0
Alphaproteobacteria	9	35.7	10
Betaproteobacteria	5	7.4	19
Gammaproteobacteria	5	14.3	56
Deltaproteobacteria	5	21	4
Planctomycetes	1	0	1
Chloroflexi	6	0	4
Nitrospirae	3	0	26
Gemmatimonadetes	4	0	4
Verrucomicrobia	2	0	2
Firmicutes	1	0	0
SPAM	1	0	0

Discussion

I hypothesized that roots and water entering lava tubes would create a mosaic of available organic C and N, and that this mosaic would support similar variation in microbial communities. The data presented here showed substantial variation in C and N, with the Root Zone having the highest level, the Wet and Dry Floor Zones having intermediate levels and the Wet and Dry Ceiling Zones having the lowest. In contrast, microbial communities across sampling sites appeared to be similar in composition at the phylum level, suggesting that the roots, both growing into and around the lava tube may be serving as a seeding agent for microorganisms.

Nutrients

The prediction that roots entering the lava tube cave would create higher amounts of C and N in the Root Zones was supported; however, the prediction that the water entering the cave would create higher amounts of C and N in the Wet Zones was not supported. The Root Zone, which was composed primarily of root biomass and the immediately associated or connected rhizosphere, represented a localized zone of high C and N levels. Detritus from the roots was deposited on the floor directly below the Root Zones, creating an area on the floor of the cave with levels of C and N that were higher than the surrounding areas, but not as concentrated as found on the roots themselves (Table 1) . I anticipated that deposition of C and N in Wet Ceiling Zones would cause higher C and N levels in these sites than in Dry Ceiling Zones however this prediction was not supported by my data. Ceiling and Floor Zones had total organic C and N values

equivalent to other studies of comparable rock and mineral environments. In terrestrial basalt flows in Hawaii Gomez-Alvarez et al. (2007) found that total C and N values were lower than 1.0% C and 0.03% N, while Northup et al. (2003) found 0.002-0.103% C and 0.01-0.04% N in the soils and ferromanganese crust of Lechuguilla Cave. However, both Floor Zones exhibited higher levels of C and N than both Ceiling Zones. Individual water droplets falling through the void of the cave and splashing apart on the floor of the lava tube could produce such a result. This result suggests that cave floors could support a unique and slightly enriched community of microbes, one that is regularly missed by cave researchers, since most samples are taken from ceiling and wall sites.

Diversity of Microbial Communities

The absence of significant differences in diversity among the Root, Wet Ceiling, Wet Floor, Dry Ceiling and Dry Floor Zones at the 97% sequence similarity level (Table 2) indicated exposure to roots and water did not create areas of more complex microbial communities. Chao estimate curves suggested that Root, Wet Ceiling and Dry Ceiling had slightly more diversity than the Wet and Dry Floor Zones. However, the upward trend in the Chao estimate curves (Figure 5) suggested that diversity was still dependent on sampling. Although the results were not significantly different, Shannon's index results showed that Root (H'=4.65), Wet Ceiling (H'=4.25) and Dry Ceilings (H'=4.11) had higher diversity than Wet (H'=3.72) and Dry Floors (H'=3.95). Chao estimates and Shannon's index results indicated neither exposure to percolating water nor the higher nutrient levels of the Floor Zones increased diversity, suggesting that something other

than nutrient levels are driving the diversity levels of the microbial communities (Table 2, Figure 5). In comparison to two other similar habitats, all five zones had higher diversity than terrestrial basalt deposits in Hawaii (H'=3.5; Gomez-Alvarez et al., 2007) and a cave sampled in Slovenia (H'=3.19; Pašić et al, 2010). While there was no significant difference in diversity at the 97% sequence similarity level, at the 80% sequence similarity level the Root Zone showed a significantly higher level of diversity than the other four zones, suggesting that the microbial communities of the roots are more diverse at the phylum level and that additional deep sampling may show this increased diversity at the 97% sequence similarity level (Table 2).

Distribution of Phyla and Possible Metabolic Pathways

The prediction that Root and Wet Ceiling and Floor Zones would have higher percentages of clones that grouped with phyla common in soils, such as *Alphaproteobacteria* and *Bacteroidetes* was supported for the Root Zone but only partially supported for areas with exposure to only water percolating from the surface. While the Roots appear different from the other zones, there were few differences among the Wet and Dry Ceiling and Floor Zones. Root Zones had a higher percentage of clones from the phyla or sub-phyla *Alphaproteobacteria*, *Bacteroidetes* and *Chloroflexi* than all other zones (Figure 6). The *Alphaproteobacteria*, which include numerous plant symbionts, are common in soils and can cope with hostile environments that have extremes in pH, temperatures, salinity and nutrient levels (Madigan et al., 2009; Rastogi et al., 2010). The phylum *Bacteroidetes* is common in soils, sediments and seawater, but

the most studied relatives are important in gut processes of mammals, while the *Chloroflexi* are phototrophic and organotrophic bacteria found in numerous environments (Madigan et al., 2009). Wet and Dry Ceiling and Floor Zones had a higher number of clones obtained that grouped within the *Gamma-* and *Deltaproteobacteria*, *Actinobacteria* and *Acidobacteria* than the Root Zones (Figure 6). Numerous members of the *Gamma* and *Deltaproteobacteria* are chemolithotrophic bacteria (methane, sulfur and nitrite oxidizers) found in extreme environments, such as at hydrothermal vents, hot springs and ice environments, and several medically important pathogens (Madigan et al., 2009). The *Actinobacteria* phylum is common in soil environments, includes members that usually prefer stable, non-extreme environments and are famous for their production of antibiotics, while *Acidobacteria* are a relatively new phylum, with members that are primarily acidophilic and found regularly in culture-independent studies of soils (Madigan et al., 2009). No other major patterns in the phyla of the clones appeared according to nutrients present or with the Ceiling and Floor Zones; however, Dry Ceiling and Floor Zones did have fewer clones that grouped within the *Actinobacteria* than the other zones, consistent with their generally poor tolerance of desiccation. Except for differences in percentage of clones that grouped within the *Actinobacteria,* the microbial communities of the Ceiling and Floor Zones were similar, which was expected since substrates of each of these zones are basalt rock or soils most likely formed of broken down basalt. The Root Zone is a sampling of actual root tissues, which are known to shape the microbial communities of the rhizosphere to be different from even nearby soils in other organic soil environments (Berg and Smalla, 2009; Farrar et al., 2003).

Because of this, it is not surprising that the Root Zone was different from the other four zones.

Clones that are closely related to cultured relatives with known metabolic abilities can help elucidate possible metabolic pathways and energy sources present in these cave systems. Sulfur oxidizers and reducers from the *Proteobacteria*, mainly from the *Beta-*, *Gamma-*, and *Epsilon-* subphyla, were found in Frassasi, Altamira and Movile Caves (Chen et al., 2009; Engel et al., 2004; Macalady et al., 2006; Portillo et al., 2009a). Northup et al. (2003) found evidence of putative nitrogen cycling and iron and manganese oxidizing bacteria from the *Proteobacteria* in Lechuguilla Cave. Evidence of both chemoorganotrophs and chemolithotrophs were found in Roots Galore and Pahoehoe Caves (Table 5). Two clones from the Root Zone were found to be closely related to known iron and manganese oxidizers *Hyphomicrobium hollandicum* and *Pedomicrobium australicum* (Gebers and Beese, 1998; McDonald et al., 2001), while one clone in Dry Floor Zones grouped with a known manganese oxidizer (Kim et al., 2006). Three clones from Root, Wet Ceiling and Dry Floor Zones were closely related to cultured nitrogen cycling bacteria (Daims et al., 2001; Ehrich et al., 1995; Farhbach et al., 2008;), while four clones grouped with known sulfur oxidizers/reducers (Table 5; Davis-Belmer et al., 2007; Sorokin et al., 2007; Vandiekan et al., 2006). Two clones from the Root and Wet Ceiling Zones grouped with cultured methanotrophs, which use chloromethane present in the air (Dedysh et al., 2004; McDonald et al., 2001). While closest relatives cannot conclusively predict the metabolic pathways that operate in these

study caves, they can provide suggestions that can be pursued with culturing and other studies.

Habitats of Closest Relatives

Closest relatives of sequenced clones can provide important clues to the role that microorganisms may be playing in a given environment or may indicate sources of colonization. The prediction that roots will have a higher percentage of closest relatives from other rhizosphere sites was supported for the Roots and Dry Ceiling Zones. However, the prediction that roots would have a higher percentage of clones with closest relatives from other soil studies and a lower percentage from other cave studies was not supported (Figure 8). Previous studies have found certain sequences that are common members in numerous cave environments, suggesting the possibility of cave-adapted microbial communities (Hathaway, 2010; Northup et al., 2008, Snider et al., 2009a); however, none of the microbial communities in the different zones in this study appear to contain more cave-adapted closest relatives than the other zones. The lower percentage of clones that had closest relatives from other cave and rhizosphere studies than soil environments is likely due to the paucity of published sequences from rhizosphere and cave studies in GenBank® compared to published sequences from soil studies. These results agreed with diversity and percentage of phyla findings discussed above by suggesting that the microbial communities are similar in function and cave adaption level. This means that all the zones were probably seeded from similar sources, such as roots present above and around the cave and from organic soil layers above the cave.

Microbial Community Composition

The prediction of the hypothesis that microbial community composition would be significantly different at each site was supported but exposure to water percolation or nutrient levels were not the driving factors for microbial community composition. However, the presence of roots and the specific cave from which the samples were collected appeared to be a weak driving factor in the microbial communities (Figure 10). Only 5% of the clones were shared among the Roots and the grouped Wet and Dry Ceiling and Floor Zones at the 97% sequence similarity level. Libshuff, parsimony, weighted and unweighted UniFrac comparisons showed that the microbial community composition of the Root, Wet Ceiling, Wet Floor, Dry Ceiling and Dry Floor Zones microbial communities were different from each other ($P<0.001$ to $P<0.0001$) at the 97% sequence similarity level. Since the zones have not been sampled to saturation, differences in the communities could be the result of incomplete sampling. Given that similarity of the Wet and Dry Ceiling and Floor sites at the phylum level, additional sampling may show greater similarity between these four zones.

According to the maximum parsimony trees (Figures 11, 12, and 13), clones from all six zones intermixed and no clear groupings or clades were found to stand out on their own according to exposure to roots or percolating water, suggesting intermixing and similarity in the microbial community composition of different zones. PCoA diagrams and Jackknife diagrams showed that the Root Zones of each cave did cluster together, suggesting that the microbial communities are similar to each other. However, the Root

Zones also clustered with Dry and Wet Floor sites, meaning that the microbial communities of the roots were as similar to two floor sites as to each other, further supporting the idea that the microbial communities throughout the cave are similar and probably seeded from the same source. No other clustering was noticeable, showing that microbial communities were not controlled, or only weakly controlled, by nutrient level or water percolation. Only weak clustering according to the specific cave from which the sample was collected was evident according to principal coordinate 1 (Figure 10). This weak clustering was the only suggestion in this study that the different cave locations could be a possible driving factor in microbial community composition, although Hathaway (2010) found cave location was the main driving force in microbial community composition in caves in the Azores and Hawaii.

The use of culture-independent molecular techniques has led to an increased knowledge of the diversity (Hugenhotlz et al., 2002) and complexity of caves' microbial communities (Northup et al., 2008; Portillo and Gonzalez, 2009). However, culture-independent studies are not without their limitations (Donachie et al. 2007), one of the most significant of which is incomplete sampling. The paucity of complete sampling of the diversity of all zones in this study was evident from rarefaction curves of both Root and Ceiling and Floor Zones, which appeared to be almost linear at the 97% sequence similarity levels, and appeared to reach asymptote only at the 80% levels, the assumed phylum level (Figure 4). Good's Coverage results further indicated that the full diversity of the Roots, Ceiling and Floor Zones had not been reached and that more sequencing was needed (Table 2). Thus, my data show significant differences in microbial diversity

in caves but like other studies (Hughes et al., 2001; Pašić et al., 2009; Portillo and Gonzalez, 2009), do not achieve sampling saturation. While all conclusions were supported statistically with 95% confidence level, due to the incomplete sampling, these phylogenetic results should be viewed as trends and directional generalities rather than firm conclusions.

Comparison to Other Rhizosphere Studies

Comparing the phylum level results of this study to other root and rhizosphere studies allows me to investigate whether there are groups of bacteria that are common to all rhizosphere habitat regardless of the plant species or surrounding soils (Table 3; Dimitrui and Grayston, 2009). Nearly one-fourth of the clones in this study with root-related closest relatives were related to clones from a study of bacteria associated with trembling aspens, (Lesaulnier et al., 2008), while eight clones were grouped with bacteria found in the rhizosphere of cotton (Wang et al., 2007). Eleven of the root-related closest relatives were cultured relatives, including *Mucilanginibacter* sp., *Rhizobium* sp. and *Bradyrhizobrium* (Table 3). Clone PH01-4c03 from Root Zone was related to *Agrobacterium rhizogenes*, a bacterium that causes infections and hairy root growth in roots (Table 3; Giri et al., 2001). Clones from Wet Ceiling and Dry Floor Zones were also related to *Pseudonocardia acaciae*, which is commonly found in the rhizosphere (Duangmal et al., 2009) and *Microlunatus ginsengisoli*, a bacterium cultured from the rhizosphere and soil in a ginseng field in Korea.

Differences at the phylum level between my study and the study of microbial communities associated with trembling aspens suggested that either the plant species or exposure to the cave could be affecting the microbial communities found on the roots. Lesaulnier et al. (2008) sampled clone libraries of trembling aspen trees raised in ambient levels of CO_2 and found that the rhizosphere communities contained 19 phyla, while the Root Zone of this study contained only 15 phyla (Table 6). The two tree roots had similar percentages of *Alphaproteobacteria*, *Planctomycetes* and *Gemmatimonadetes*. The ponderosa pine roots in this study had a higher percentage of clones that grouped within the *Actinobacteria*, *Bacteroidetes*, *Gammaproteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Nitrospirae*, *OP10* , and *TM7* than the trembling aspen roots (Table 6; Leaulnier et al., 2008). Trembling aspen roots had a higher percentage of clones that grouped within the phyla *Acidobacteria*, *Verrucomicrobia*, *Betaproteobacteria*, and *Deltaproteobacteria*, while *Firmicutes*, *OD1*, *Thermomicrobia*, and *Dehalococcoides* were only found on the trembling aspen roots (Table 6; Lesaulneir et al., 2008). These two tree studies were conducted in very different soils and bedrock types and different plant species, both factors that affect microbial communities of the rhizosphere (Berg and Smalla, 2009, Carter et al., 1999; Dennis et al., 2010). While these differences are the result of some driving force on the microbial community, including exposure to the cave, species of the plant roots or soil properties, determining the nature of this driving force is beyond the scope of this study. However, this is one of the few studies of microbial communities associated with non-crop roots, showing the need for these kinds of studies.

Comparison to other Cave Studies

Comparisons between this study and other cave microbial community studies could suggest the existence of a cave adapted microbial community with common members found in different caves worldwide. Several clones from the lava tubes were closely related to environmental clones found in other cave environments (Table 4). Fifteen clones from this study grouped with clones from an unpublished study in the Oregon Cave National Monument, and 10 of them grouped to the same clone, 17N118. Furthermore, nine sequences grouped with clones from a Slovenian cave and six of these grouped with the same clone, clone 2PJM54 (Table 4). In addition, several clones had both cave and rhizosphere-associated closest relatives, such as Clone RG25-f07 and Clone RG28-c08, further suggesting roots serve as a vehicle of colonization into shallow caves (Figure 11).

While lava tubes and limestone caves are both cave environments, resulting in some similarities, their parent rocks and nutrient levels are different. This resulted in phylum level differences between lava tube and limestone cave microbial communities, evident in the following example comparisons between the grouped Ceiling and Floor Zones from this study, white colonies from Altamira Cave in Spain and colonies from Pajsarjeva jama Cave from Slovenia (Table 7). The grouped Ceiling and Floor Zones clone libraries included 14 phyla, making it the most diverse at the phylum level, while the Altamira Cave study found 5 phyla and the Slovenian cave study found 10 phyla. White colonies from Altamira Cave had a higher percentage of clones that grouped within the Alphaproteobacteria, *Betaproteobacteria*, *Gammaproteobacteria*, and

Deltaproteobacteria (Table 7). *Alphaproteobacteria* and *Gammaproteobacteria* were the dominant metabolically active bacteria in white, yellow and grey colonies in Altamira Cave (Portillo et al., 2008; Portillo and Gonzalez, 2009). The white colonies in Altamira Cave had a lower percentage of clones that grouped within the *Acidobacteria*, while no clones were found that grouped within the *Actinobacteria, Bacteroidetes, Planctomycetes, Chloroflexi, Nitrospirae, Verrucomicrobia, Firmicutes, SPAM*, and *Gemmatimonadetes,* showing that the lava tube caves in this study were much more diverse at the phylum level (Portillo and Gonzalez, 2009). Similar percentages of clones from Ceiling and Floor Zones and the Slovenian Cave grouped with *Alphaproteobacteria, Deltaproteobacteria, Planctomycetes, Gemmatimonadetes,* and *Verrucomicrobia*, while a higher percentage of clones from *Acidobacteria* and *Chloroflexi* were found in the Ceiling and Floor Zones than in the Slovenian Cave. Pajsarjeva jama Cave had higher percentages of clones from *Actinobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Nitrospirae* than on the Ceiling and Floor Zones, while no clones from the Slovenian Cave grouped within the *Bacteroidetes, Firmicutes*, and *SPAM*, all of which were represented in the Ceiling and Floor Zones These comparisons show that while the limestone cave and lava tube caves have similar phyla represented in both, differences exist in the number of phyla present and the percentage of clones that group within each phyla in each type of cave (Table 7).

Implications of this Study and Conclusions

This study is one of the first to look at microbial communities throughout the lava tube environment, offer evidence on how plants on the surface affect lava tubes and provide preliminary suggestions on how any shallow cave, lava tube or limestone, is affected by the surface and seeded with microorganisms from the soils and roots near the cave. Protection of any shallow cave or lava environment should include management and protection of the vegetation above and near the cave system, given that plant roots act as conduits between the surface and subsurface both directly and indirectly. This research also allows for a better understanding of the composition and source of microbial communities in lava tubes, which are currently viewed as possible analogs for Martian astrobiology research. Given that lava tubes, and all shallow caves, are greatly influenced by and probably receive many of their microbial inhabitants from surface sources, microbial communities in Earth lava tubes could appear very different from that found on Mars. Assuming that lava tubes below surfaces with significant plant growth and soils above them, such as on Earth, might be analogous environments to lava tubes on a barren Martian surface should be done with caution. In addition, results from this study have already been used to help research and isolate areas that may prove fruitful in the hunt for antibiotic producing *Actinobacteria*. This study showed that ideal habitats for *Actinobacteria* were on continually wet walls and ceilings in lava tubes. Finally, this study is one of the first *in situ* studies of how roots affect the soils and voids that they occupy and grow near. Differences between the microbial communities in soils with and without direct contact to plant roots may be smaller than expected.

This study found that there were significantly higher amounts of C and N in areas with exposure to roots. However, there were no significant differences among the Root and Wet and Dry Ceiling and Floor Zones in diversity, in the percentage of clones that were related to cave, rhizosphere or soil closest relatives or in phylum-level distribution. All zones had clone libraries that were a mixture of cave, rhizosphere or soil-related isolates and all zones appeared to have both chemoorganotrophs and chemolithotrophs. In addition, differences seen at the 97% sequence similarity level appeared to be random, expect for similarity between the roots of each cave, showing no significant similarities according to zone. This suggests that the presence of roots caused only minor differences in the microbial community composition and presence of percolating water had no effect on microbial community composition. Additionally, differences in microbial communities in the caves at the 97% sequence similarity level could be over-represented since sampling saturation was not achieved. This conclusion is supported by the fact that communities at the 80% level are similar and that certain aspects of the microbial composition and properties, such as percent of clones with cave, soil or rhizosphere-related closest relatives, appeared to be similar throughout the lava tube cave. Indications are that the microbial communities throughout the two caves could be shaped by the same factors and seeding agents, primarily roots and soils, found around the lava tube cave. If a cross-section of the cave within the soil and rock could be displayed, the cave would not be an isolated structure. Surrounding it from above, to the sides and around the bottom are roots, rhizosphere, and soils that had filtered down and basalt bedrock. Microbes from these roots and soils migrated out of their original environments

and affected the microbial communities of the lava tube Ceiling and Floor environments. Therefore, communities on the cave Ceiling and Floor Zones and in the Root Zone shared similar community attributes and members, since they all came from microbial communities from the same roots and soils. Concisely stated, the microbial communities in these lava tube caves, and possibly all shallow caves, come from the soil and plant roots above and around the cave.

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