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Molecular phylogenetic investigation of microbial diversity and nitrogen cycling in lava tubes

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MOLECULAR PHYLOGENETIC INVESTIGATION OF MICROBIAL DIVERSITY AND NITROGEN CYCLING IN LAVA TUBES

BY

JENNIFER J. MARSHALL HATHAWAY

B.S., BIOLOGY, STANFORD UNIVERSITY 2001

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Masters of Science
Biology

The University of New Mexico
Albuquerque, New Mexico

May, 2010

DEDICATION

This work is dedicated to my parents, Bill and Jane Marshall, who taught me at a very young age to see the wonder of the natural world, and to ask questions about the way it works.

And to my husband, Simon Hathaway, who teaches me every day about finding joy in both the little and big moments in my life. Thank you for allowing me to have this adventure.

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Lurdes and Airidas Dapkevicius provided support and generous hospitality while I was in the Azores, and I thank them for their help, and their friendship.

I want to thank my family and my husband's family for encouraging me to undertake this endeavor from the start, and supporting me every step of the way.

Finally, I want to thank my husband, Simon, for his never ending love and support in whatever project I am undertaking. He listened patiently to my explanations of my research, and helped me find ways to overcome any obstacle in my path.

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ABSTRACT

Worldwide, lava tubes host colorful microbial mats on their walls and ceilings. Little is known about the diversity and ecological roles of the bacteria communities in the subsurface ecosystem of lava tubes. White and yellow microbial mats were collected from four lava tubes from the Azorean island of Terceira and from four lava tubes on the Big Island of Hawai‘i, to compare and contrast the diversity of bacteria found in lava tubes. The 16S rRNA gene was sequenced in order to determine the diversity within these caves, and to begin to elucidate the environmental controls on microbial diversity in the subsurface by comparing community structure to environmental parameters. One hundred ninety two sequences from 16 samples were analyzed. Fifteen phyla were found across the samples. With more *Actinobacteria* clones retrieved from Hawaiian communities, while more *Alphaproteobacteria* clones were found in Azorean communities. The *Actinobacteria* exhibited considerable novel diversity, with several

distinct novel clades that shared less than 94% sequence identity. Geographical location was the major contributor to differences in community structure.

The diversity of ammonia oxidation (*amoA*) and nitrogen fixation (*nifH*) genes in bacterial mats from lava tube walls in the Azores was investigated using denaturing gradient gel electrophoresis (DGGE). The lava tubes were found under different land use categories, pasture, forested and sea/urban. Soil and water samples from each lava tube were analyzed for nutrient content. *Nitrosospira*-like sequences dominated the ammonia oxidizing bacteria (AOB) community, and the majority of the diversity was found in lava tubes under forested land. The nitrogen fixation community was dominated by *Klebsiella pneumonia*-like sequences, and diversity was evenly distributed between pasture and forested land. The results suggest that land use is impacting the AOB more than the nitrogen fixing bacteria. Furthermore, the results of these studies underscore the need for further investigation of these unique ecosystems.

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Chapter 1

Introduction

Extreme environments that harbor microbial life are found on the surface and in the subsurface across the planet, from sulfur hot springs to the salt flats to dry deserts to the hydrothermal vents at the bottom of the ocean (Rothschild and Mancinelli, 2001). These microbes play important roles in shaping the ecosystems they inhabit. Microbes are central in cycling nutrients through the precipitation and dissolution of minerals, both at the surface (Ehrlich, 1999) and in the subsurface (Northup and Lavoie, 2001; Barton and Northup, 2007).

Caves and lava tubes often have moderate temperature and relatively stable humidity throughout the year, but are still considered extreme environments because of their low nutrient availability and productivity (Moore and Sullivan, 1997; Northup and Welbourn, 1997; Barton, 2006). The microorganisms of limestone caves have received greater scientific attention in the last 20 years, while lava tubes and their microbial communities have received far less attention (Northup and Welbourn, 1997). Parenthetically, the recent evidence of volcanic activity and lava tubes on Mars has renewed interest in studying the lava tubes found on Earth and it is thought that if there is any evidence of life on Mars, it will be found in the lava tubes, as they are relatively removed from harsh surface conditions (Boston et al., 1992). Recent preliminary studies suggest that the microbial diversity of the biofilms found in lava tubes is much higher than previously thought (Northup et al., 2008; Garcia et al., 2009; Moya et al., 2009; Snider et al., 2009).

The traditional way to study bacteria is to isolate them in culture. These techniques limit the complete study of most environmental samples, primarily because of lack of understanding of the combination and concentration of nutrients and other essential minerals and conditions required for bacterial growth (Rusterholtz and Mallory, 1994; Northup et al., 1994; Amann et al., 1995; Hugenholtz et al., 1998).

The development of better DNA sequencing methods has allowed for new ways to isolate and classify organisms. The most widely used scheme for prokaryotes is based on sequences of 16S rDNA. The application of these culture-independent (16S SSU) molecular biology techniques to environmental samples revolutionized the way microorganisms are identified *in situ* and led to the discovery of extensive novel diversity in the microbial communities in many environmental samples (Pace, 1997). Applying this and other culture independent methods to cave and lava tube samples allows us to start untangling the mysteries surrounding the ecology of microorganisms in cave systems, and lava tubes in particular.

Colorful microbial mats on the walls of lava tubes have been known for some time (Howarth, 1981), but it is only recently that the extent of biodiversity contained in these mats has been revealed. They occur in varied climates, from the dry deserts of New Mexico to the humid hillsides of Hawai‘i, to the temperate climates of the Azorean Islands and Canary Islands. Recent studies of microbial mats suggest there are similar phyla found in the lava tubes of New Mexico and Hawai‘i (Moya et al., 2009). However, an in depth analysis of species similarity between the two sites was not conducted, leaving the question, is there a common community of bacteria in lava tubes worldwide?

Or is each lava tube unique, with local environmental and geochemical factors influencing the bacterial community?

Knowing the overall bacterial diversity within the lava tubes gives part of the picture. Understanding what types of metabolic processes are occurring within lava tubes would allow better comprehension of the ecological role the bacteria play within these unique environments. One such ecological role is the cycling of nitrogen. The global nitrogen cycle is strongly influenced by microbial processes (Bock and Wagner, 2006). After carbon, nitrogen is the most abundant element in terms of biomass. Two critical steps in the nitrogen cycle, nitrogen fixation and ammonia oxidation, are performed exclusively by microorganisms. Nitrogen fixation by bacteria can be the main source of bioavailable nitrogen in many environments, including soil and oligotrophic oceanic waters. Bacteria can metabolize ammonia as their sole source of energy in lithotrophic environments. The organisms responsible for these nitrogen transformations have been well studied in soil and aquatic habitats, yet new discoveries are still being made (Venter et al., 2004; Francis et al., 2007; Prosser and Nicol, 2008). These analyses have just begun to be applied in subterranean habitats where nitrogen limitation is thought to be a major constraint on biological production (Barton and Northup 2007). Recent 16S rDNA diversity studies have suggested the presence of ammonia oxidizing bacteria in Spider Cave in New Mexico and El Malpais lava tubes (Dichosa, 2008; Moya et al., 2009). It is known that certain *Actinobacteria* can fix nitrogen, and this phylum of bacteria is frequently found in caves and lava tubes (Northup et al., 2003; Barton et al., 2004; Northup et al., 2008). Two studies to date have looked for the presence of *amoA*, a gene involved in ammonia oxidation, in the subsurface (Spear et al., 2007; Chen et al., 2009).

However, neither of these studies was conducted in lava tubes. Furthermore, the nitrogen fixation gene, *nifH*, has not been investigated in subsurface environments. Studying the presence and diversity of these two genes in lava tubes would help in our understanding of the metabolic processes occurring in these ecosystems.

The main goals of this research were:

1. *To investigate the influence of geography and other abiotic factors on microbial community structure in lava tubes of Hawai'i and the Azores.* The study aimed at examining if there is a global community of bacteria that inhabit lava tubes, or if local environments control the microbial diversity. Results of this study are presented in Chapter 2.
2. *To investigate the presence of two nitrogen cycling genes in lava tubes under different land use categories*. This research only focuses on the lava tubes of Terceira Island, Azores. Water and soil chemistry were also measured as indicators of carbon and nitrogen content in lava tube material. I hypothesized that different nitrogen inputs would be a result of the different land use categories, and would result in different diversity of ammonia oxidizing and nitrogen fixing bacteria in the underlying lava tubes. Results of this research are presented in Chapter 3.

Together the research put forth in this thesis furthers the understanding of microbial communities in lava tubes worldwide. Understanding what types of bacteria occur in these ecosystems lays the groundwork for future studies of more specific aspects of microbial life in lava tubes and other cave environments.

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Chapter 2

Investigation of Novel Microbial Diversity in Azorean and Hawaiian Lava Tubes

Introduction

Lava tubes, which are caves formed by magma flows, develop microbial mats that cover walls and ceilings with remarkable colors and patterns. These mats are commonly found in moist lava tubes, however very little is known about their diversity and the role they play in the biogeochemistry of this subsurface environment. Notably, they have received less attention than the epilithic microorganisms of limestone caves (Northup and Welbourn, 1997; Northup et al., 2008; Garcia et al., 2009; Moya et al., 2009). Lava tubes, like other caves, often have relatively stable temperatures and humidity throughout the year once into the dark zone (Palmer, 2007), but are still considered extreme environments because of their low nutrient availability and productivity (Moore and Sullivan, 1997; Northup and Welbourn, 1997; Barton, 2006). Culture-dependent techniques have been the common method applied in studying the composition of these mats, often referred to as "slimes". Stoner and Howarth (1981), the first to describe these mats in Hawai'i, found fungi and aerobic bacteria in Hawaiian lava tubes and suggested that the white and brown slimes are important sites of nutrient cycling (e.g., nitrogen). Studies done in lava tubes in Washington, USA, have found slime consisting of different species of bacteria, including *Actinobacteria* in the genus *Streptomyces* (Staley and Crawford, 1975). Biotechnological interest in these microbial mats has been increasing because certain types of *Actinobacteria* excrete antibiotic products (Lazzarini et al., 2000). Recent studies of other types of volcanic terrain have also reported novel chemolithotrophic bacteria (Gomez-Alvarez et al., 2007; King, 2007; Stott et al., 2008). *Acidobacteria*, *Alpha-* and *Gammaproteobacteria*, *Actinobacteria*, and *Cyanobacteria* dominate bacterial communities on volcanic surface terrain in Hawai‘i, and it appears

that composition is controlled by local differences in the environment and composition of the volcanic deposits (Gomez-Alvarez et al., 2007).

Recent evidence of volcanic activity and lava tubes on Mars has renewed interest in studying the lava tubes found on Earth and it is thought that a likely environment in which to find evidence of fossil or extant life on Mars would be lava tubes, as they are relatively removed from harsh surface conditions (Boston et al., 1992; Boston et al., 2001). Despite these motivations, little is known about the diversity and development of microbial communities in lava tubes in relation to environmental factors both inside and outside the lava tube. From a biogeographic perspective, it is not clear whether there is a microbial community structure common to lava tubes worldwide, upholding the adage that "everything is everywhere but the environment selects" (Baas-Becking, 1934; de Wit and Bouvier, 2006), or are the bacterial communities unique within each lava tube?

To investigate these questions, we compared microbial community composition in lava tubes on the Big Island, Hawai'i, and on the Azorean Island of Terceira, which represent semi-arid to tropical and temperate climate regimes, respectively. These two islands offer a range of lava tubes with different precipitation levels and elevations. Both locations have humid lava tubes that are dominated by white and yellow-pigmented microbial mats. The Azores tubes maintain a relatively constant temperature of 15°-16°C, while the lava tubes of Hawai'i vary from 14°-19°C. Abiotic factors, such as temperature, have been shown to influence the invertebrate communities of lava tubes, but studies have not yet been conducted on the bacterial communities (Howarth, 1982; Martín and Oromí, 1988).

We hypothesized that microbial mats of similar pigmentation have a common

microbial community composition across sites. Alternatively, we hypothesized that the composition of lava tube microbial communities will vary in relation to precipitation levels, elevation, and temperature with patterns similar to those of surface microbial communities. We also hypothesize that these communities include novel organisms, sharing less than 97% similarity to known sequences in the NCBI database. Analysis of the community composition of these lava tubes will help us to further understand controls on microbial diversity and ecological organization that may extend to other habitats, possibly even other planets.

Results:

Analysis of Community Composition

A total of 1445 full-length non-chimeric sequences were obtained from the 16 libraries after screening for quality and the presence of chimeric sequences (Ashelford et al., 2005; Ashelford et al., 2006). Rarefaction curves for each location did not show saturation (figure not shown), evidence that a more extensive sampling and sequencing effort are necessary to fully describe the bacterial community present in microbial mats. These results are commonly observed in bacterial diversity studies and deep coverage of microbial communities is beyond the scope of this research.

Fifteen phyla were identified across 16 clone libraries using the Ribosomal Database Project (RDP) analysis tools after operation taxonomic units (OTU) were defined at 97% sequence similarity (Fig. 2A). The largest percentage of sequences from the Hawaiian communities were identified as *Actinobacteria* (14%), while *Acidobacteria* were most numerous in the Azorean communities (21%) (Fig. 2B).

Figure 1. Inside the Azorean (A, B) and Hawaiian (C, D) lava tubes, with visible white microbial mats lining the walls. Insets (B, D) are closer views of the microbial mats. Photographs by Kenneth Ingham.

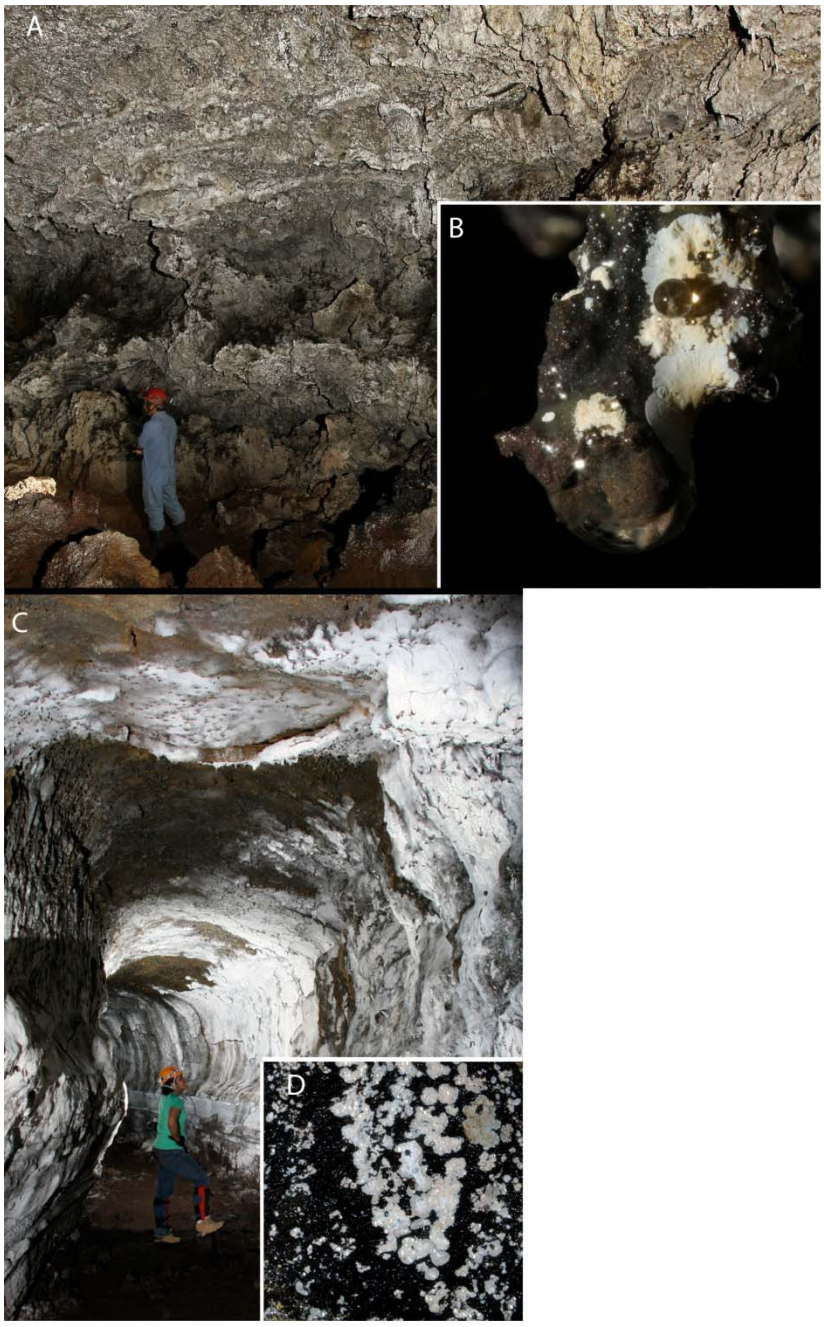


Figure 2A. Bar chart of phyla found in each lava tube based on OTUs (Operational Taxonomic Units) defined at 97% identity. B: Percent of sequences of a given phyla from each location. BP: Bird Park, EP: Epperson's Cave, KAU: Kaumana Cave, KK: Kula Kai, GBO: Gruta Branca Opala, GAS: Gruta da Achada, GP: Gruta dos Principiantes, GBL: Gruta da Balcões.

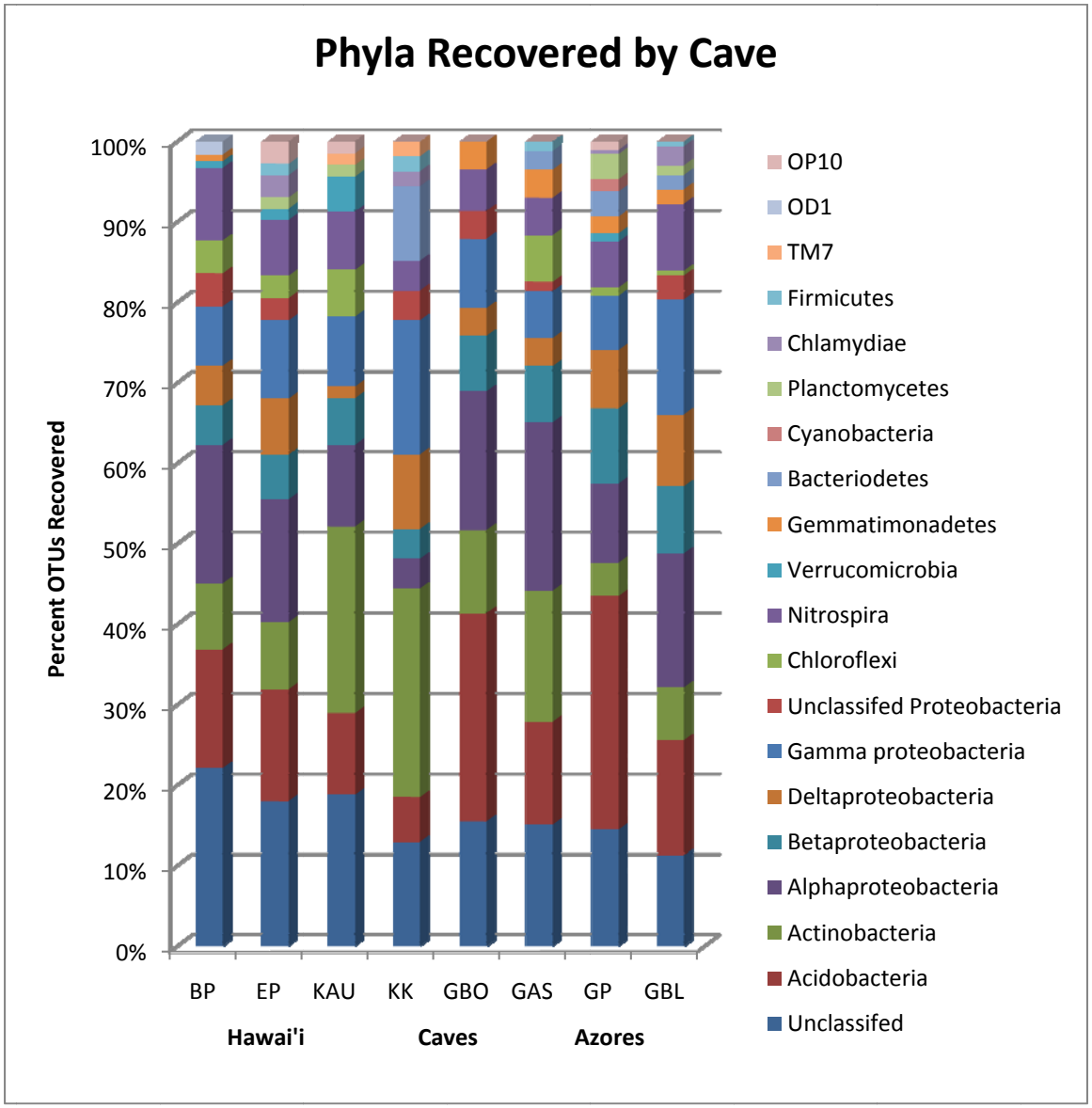
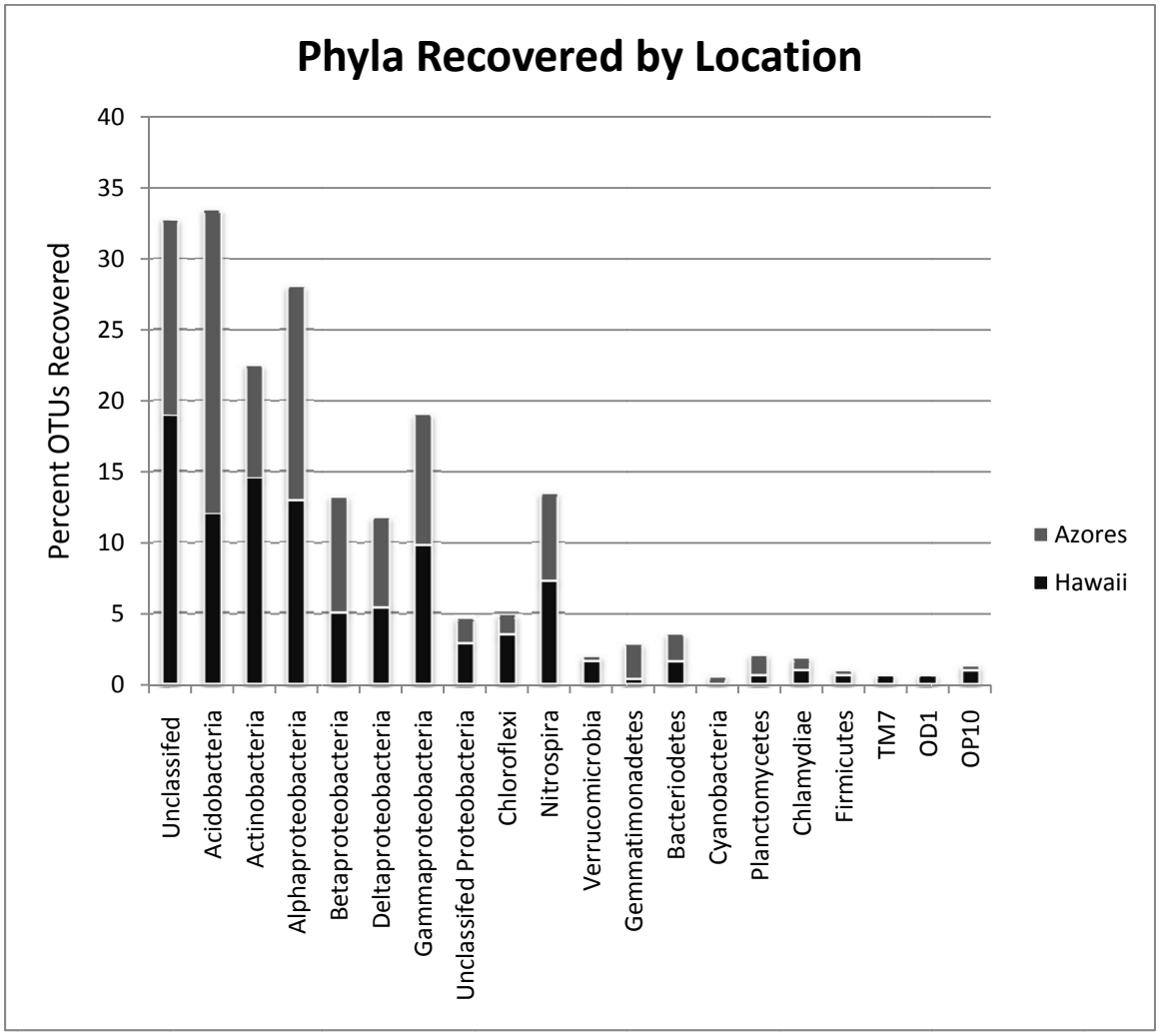


Figure 2B Phyla Recovered by Location



Alphaproteobacteria represented approximately 12% of the Hawaiian sequences and 15% of the Azorean sequences (Fig. 2A). Nineteen percent of Hawaiian sequences and 14% of Azorean sequences could not be assigned to the phylum level and were not chimeric. Other taxa comprising a substantial portion of the community include *Nitrospirae, Gammaproteobacteria, Betaproteobacteria* and *Deltaproteobacteria* (Fig. 2A). Phyla with less than 2% of the recovered sequences included *Cyanobacteria*, *Planctomyetes*, *Chlamydiae, Verrucomicrobia*, *Gemmatimonadetes*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, *TM7*, *OD1*, and *OP10* (Fig. 2A).

Analysis in a Greengenes reference tree in ARB shows that many of the nearest neighbors were from environmental soil, water, or deep-sea hydrothermal vent samples. Approximately seven percent of the defined OTUs had close matches from other volcanic environments when compared using BLAST. Nearest neighbors from other caves were also found, including Lower Kane Cave (Wyomig) (Engel et al., 2003; Engel et al., 2004), Mammoth Cave (Kentucky) (unpublished), Frasassi Cave (Italy) (Vlasceanu et al., 2000; Macalady et al., 2007; Macalady et al., 2008), Altamira Cave (Spain) (Portillo et al., 2008), Oregon Cave (Oregon) (unpublished), Nullabor (Australia) (Holmes et al., 2001), and Lechuguilla Cave (New Mexico) (Northup et al., 2003; Spilde et al., 2005).

Rarefaction curves were generated for each library using the program *mothur* (Schloss et al., 2009), and none showed that the extent of the diversity had been captured at the 97% identity level. Shared OTUs between the two sites comprised only 4% of all OTUs defined. Chao1 estimates of taxonomic richness at the 97% identity level showed that the lava tubes of Hawai‘i are more diverse than those of Terceira (Fig. 3). The Principal Component Analysis done in UniFrac (Fig. 4) showed a clustering along

Principal Component 1, which corresponds to location, and accounts for 13.6% of the variation. None of the other factors tested, color, precipitation levels, elevation, or temperature, showed clear grouping patterns. When the sequences from Hawai‘i and the Azores were grouped by location, the communities were found to be statistically different using the Libshuff function of *mothur*, ($P < 0.001$).

Analysis of Actinobacteria

The 89 OTU groups that were classified as *Actinobacteria* by RDP were examined in closer detail, because this group was an abundant phylum in the clone libraries for both sites, and *Actinobacteria* is a phylum of research interest due to their potential to produce antibiotics. Results from a comparison of these sequences to GenBank sequences show considerable novel genetic diversity, with 92% of Hawaiian sequences and 93% of Azorean actinobacterial sequences having <95% identity to those sequences in GenBank (Fig. 5). Three of the five known subclasses of *Actinobacteria* were found, *Actinobacteridae*, *Acidimicrobidae*, and *Rubrobacteridae*, with a majority of the sequences, 61 of 89, falling in the *Actinobacteridae*. Parsimony trees of the *Actinobacteridae* and the *Acidimicrobidae* and *Rubrobacteridae* were generated in Paup (Swofford, 2000; Figs. 6 and 7). A majority of the *Actinobacteria* OTUs recovered were from yellow samples (70%), and were evenly split between the Azores and Hawai‘i. The *Actinobacteridae* tree revealed a clade of 10 OTUs from one yellow sample from Kula Kai Caverns in Hawai‘i that group with themselves only. Their nearest neighbors are

Figure 3: Chao1 Estimate of Richness for Hawai‘i and Azores. Collection curves of estimated taxonomic richness as estimated by Choa1 at 97% identity of the OTU.

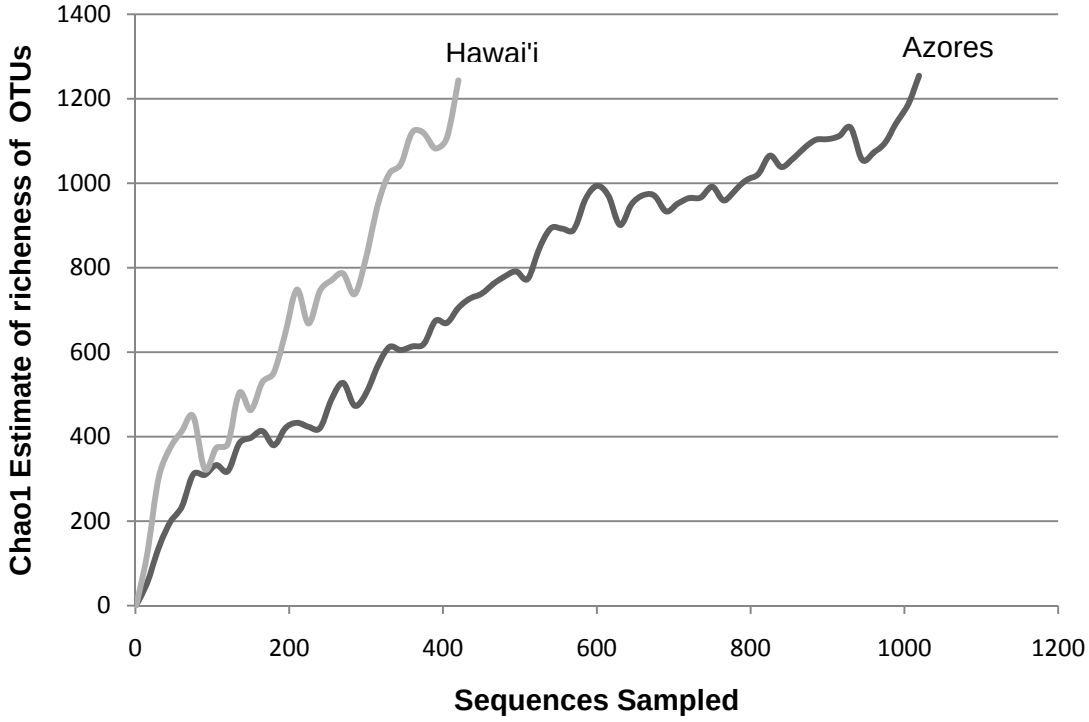


Figure 4: Principal Component Analysis of all samples in UniFrac. Clone libraries from Azores yellow samples are indicated by yellow symbols, Azores white samples are indicated by light blue symbols, Hawai‘i yellow are indicated by orange symbols, and Hawai‘i white are indicated by dark blue symbols. Names of clone libraries are listed on the right.

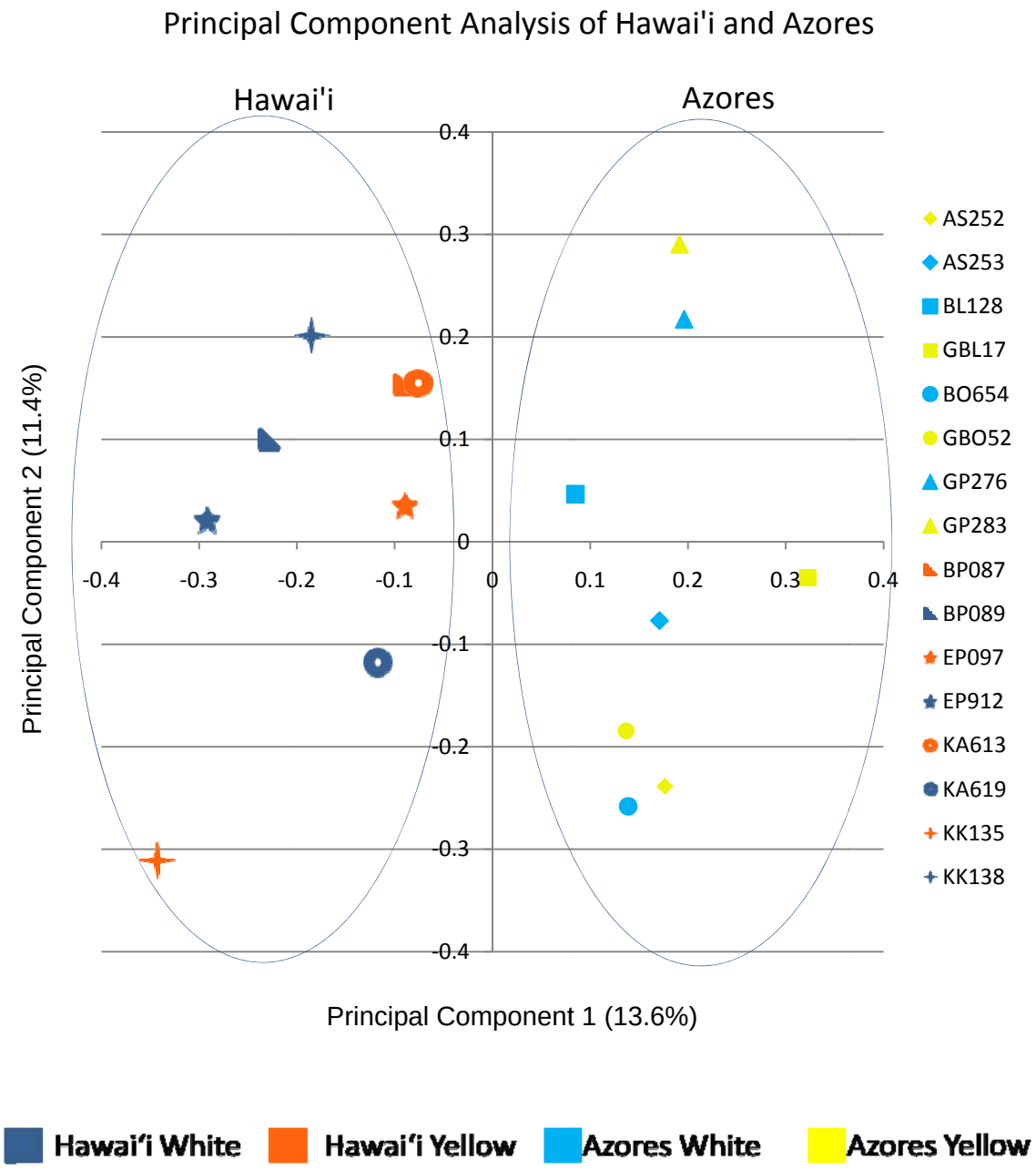


Figure 5. Sequence identity for *Actinobacteria* from both Hawai'i and the Azores based on Blast comparisons with the GenBank Database.

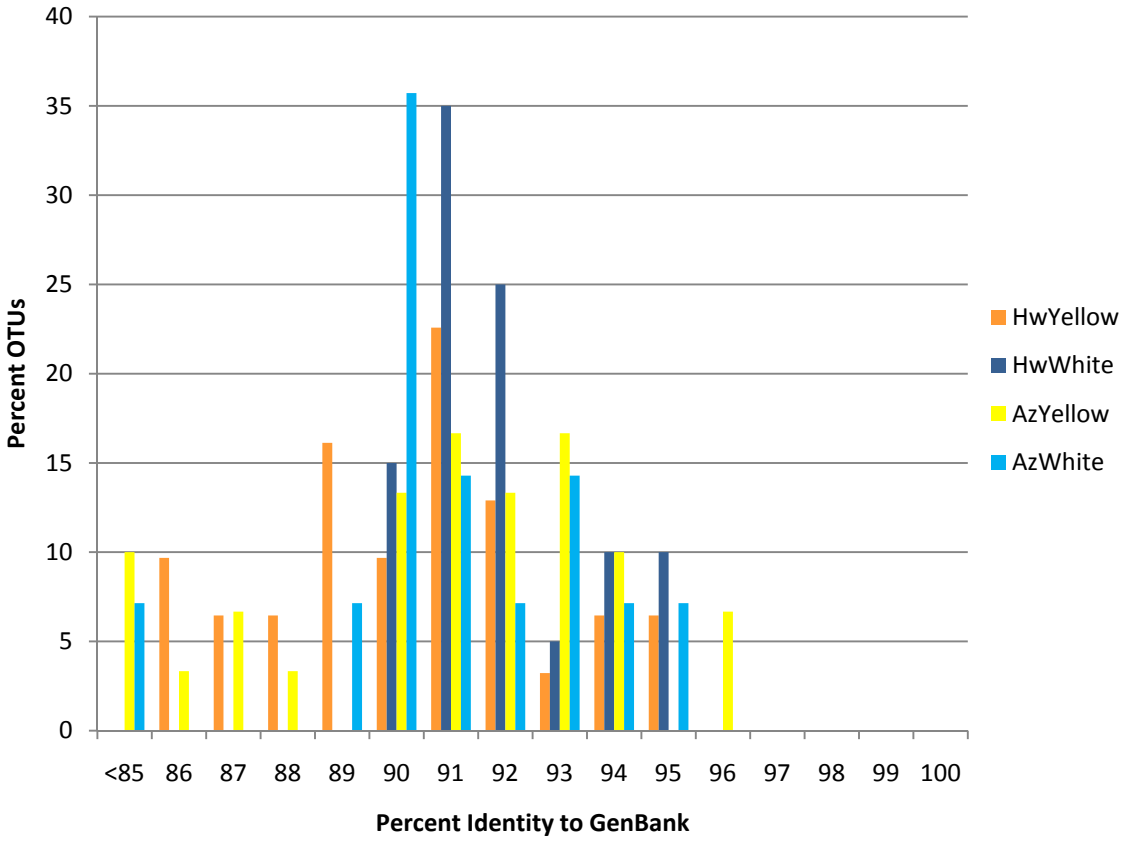


Figure 6. One of the most parsimonious trees of *Actinobacteridae*. Sequences from Azores are in bold and sequences from Hawai‘i are in bold and italics. *Actinobacteria* from other cave studies are in italic. Bootstrap values are based on 1000 replicates. Black circles indicate a bootstrap value of >75%. Grey circles indicate a bootstrap value of between 60% and 75%. Open circles indicate a bootstrap value of <60%.

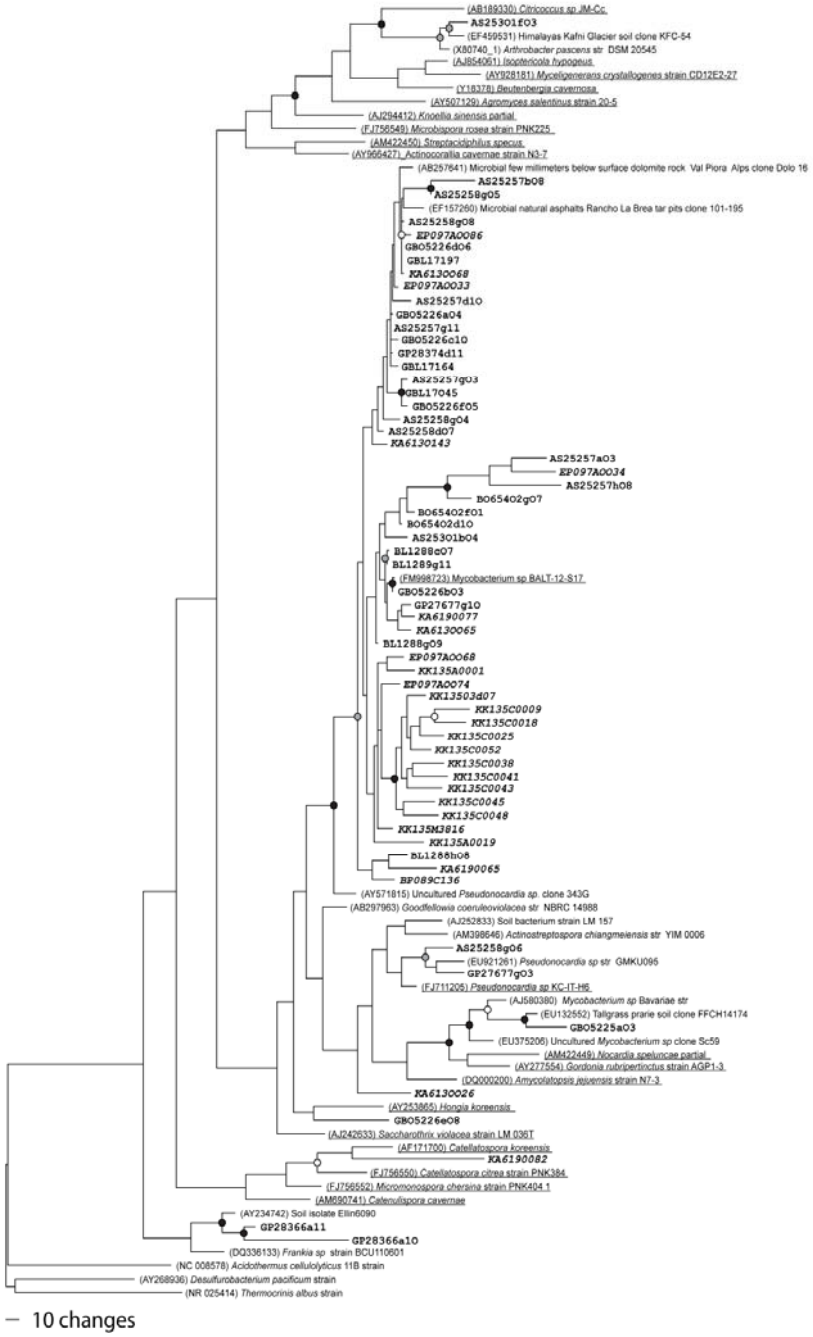
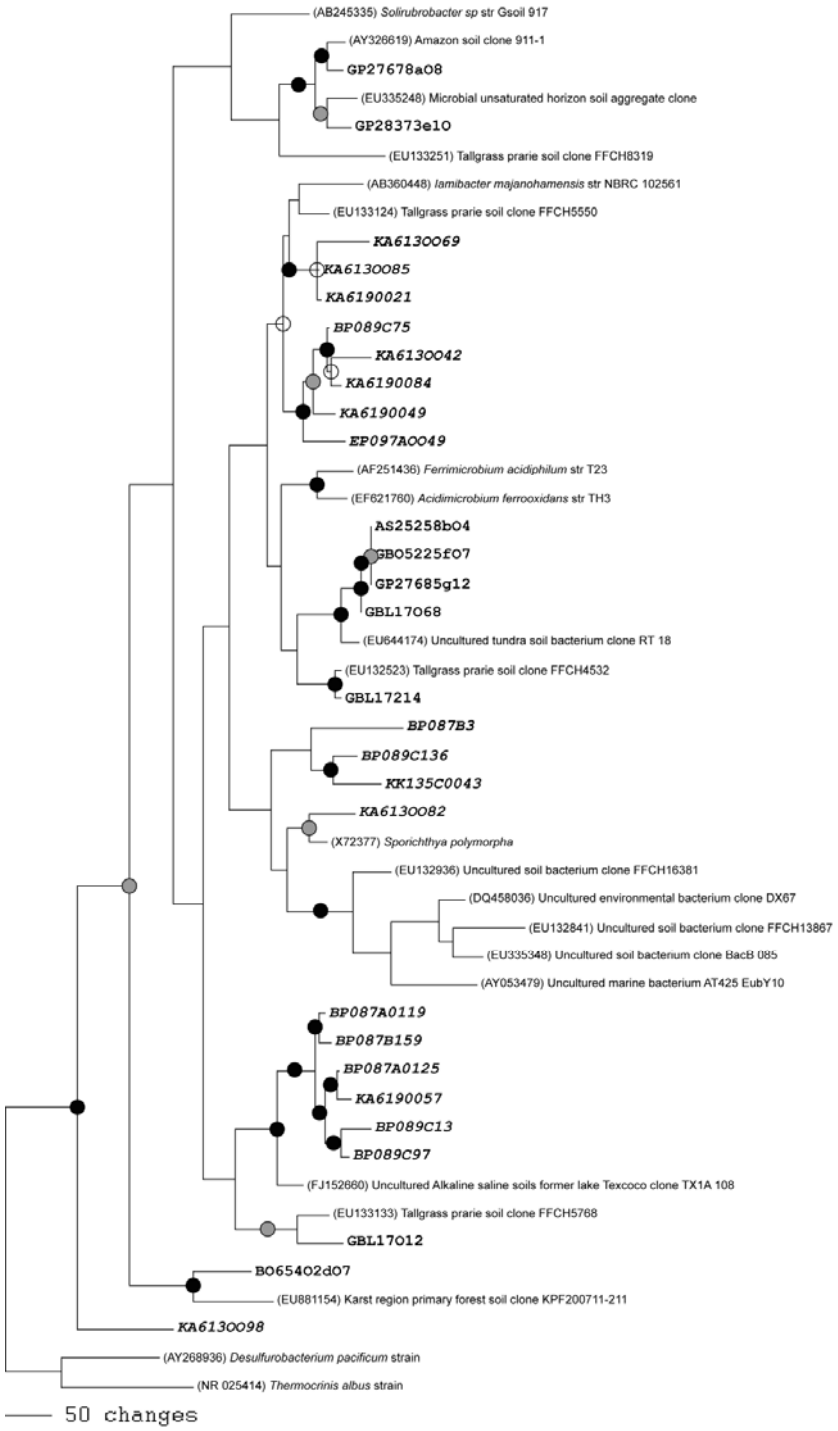


Figure 7. One of the most parsimonious trees of *Acidimicrobiales* and *Rubrobacteridae.* Sequences from Azores are in bold and sequences from Hawai‘i are in bold and italics. *Actinobacteria* from other cave studies are in italic. Bootstrap values are based on 1000 replicates. Black circles indicate a bootstrap value of >75%. Grey circles indicate a bootstrap value of between 60% and 75%. Open circles indicate a bootstrap value of 60%.



other lava tube sequences from this study, with the closest known relative being an environmental sample from dolomitic rock. Support for the position of this clade is strong, with a bootstrap value of 95%. The tree of the *Acidimicrobidae* and *Rubrobacteridae* had several clades of novel OTUs from this study with less than 97% identity to known sequences (Fig. 7). These clades tend to be from either Hawai‘i or the Azores, but rarely were there mixed clades.

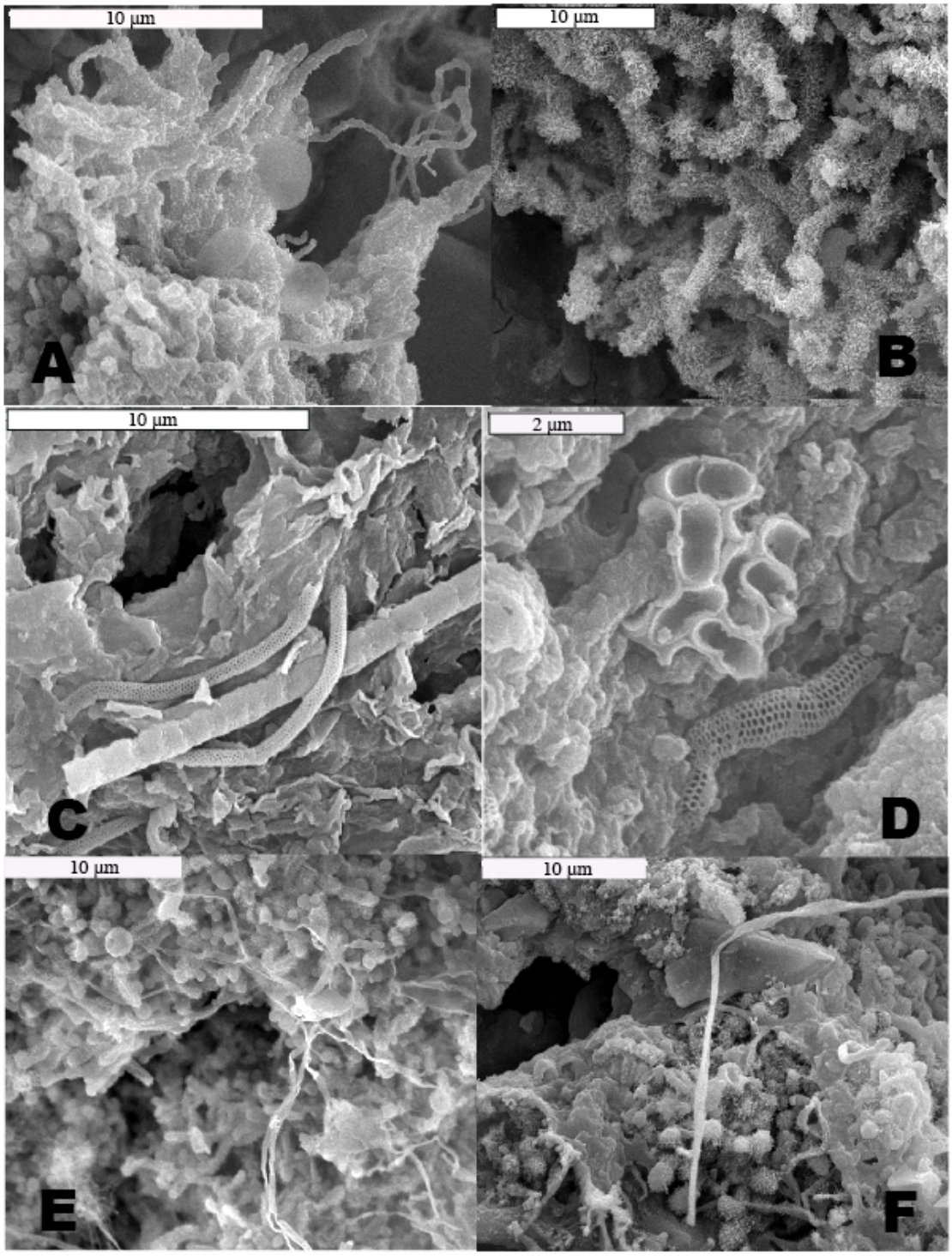
***Scanning electron microscopy*:**

Scanning electron microscopy revealed similarities and differences among the microbial communities in both locations. Coccoidal morphologies with and without abundant thread-line or knobbed appendages (putative fimbrae or pili) were detected in both locations. Reticulated filaments (Melim et al., 2008) were detected in Kula Kai Caverns (Hawai'i) and in Gruta da Balcões (Azores), as well as abundant plain, ribbon-like filamentous morphologies. Additional elongate structures with abundant thread-line appendages were observed in samples from both locations (Fig. 8). Differences observed included the presence of putative protozoa and segmented filamentous morphologies in the Azorean samples.

Discussion:

This study represents one of the first culture-independent studies of the diversity associated with microbial mats in lava tubes, and the first to compare lava tube microbial communities from different locations. The phylogenetic survey revealed at least 15 named phyla, supporting our hypothesis that lava tubes contain considerable microbial diversity. Several sequences are closely related to bacteria of non-volcanic cave habitats.

Figure 8. Similarities between Hawaiian and Azorean microbial mats as viewed with Scanning Electron Microscopy (SEM). Images on left side (A,C,E) are from Hawai‘i and those on the right (B,D,F) are from the Azores (BDF). Images A and B show arm-like filamentous morphologies with protuberances; C and D show reticulated filaments; E and F show ribbon-like filamentous morphologies.



This is probably due to the paucity of sequences from lava tubes currently in the Greengenes and NCBI databases. Seven percent of the sequences closely matched sequences from other terrestrial volcanic terrain, suggesting that there may be a common group of bacteria that live on basaltic rock both terrestrially and in the subsurface.

The Chao1 estimates of taxonomic richness show that the Hawaiian lava tubes have higher richness than the Azorean lava tubes. Hawai‘i has already been designated as a “hotspot” for prokaryotic as well as eukaryotic diversity (Donachie et al., 2004). Previous work on invertebrates and plants on the two islands suggests that this is not a unique phenomenon. When the diversity of plants and snails was compared between Hawai‘i and the Azores, it was found that Hawai‘i had considerably more diversity in both groups (Wittaker et al., 2008; Borges et al., 2009; Price, 2009).

To address the question of what drives diversity in lava tubes, we considered environmental factors such as color, precipitation, elevation, temperature and geographic location. The UniFrac PCA showed that location was the driving component of Principal Component 1, accounting for 13.6% of the variability of the data (Fig. 4). None of the other factors tested produced clear groupings, suggesting that geography is the largest predictor of community composition. This may be due to sample variability due to the limited number of clones sequenced per library. It is likely that the unique habitat of each lava tube determines the community composition, as well as dispersal constraints. In Hawaiian terrestrial lava flows, soil parameters and trace gas profiles of the lava unique to each site have been shown to influence the bacterial community composition (Gomez-Alvarez et al., 2007). Future studies should try to include additional analyses of the geochemical/mineralogical characteristics microbial mats habitat.

The founder effect may also play an important role in determining community composition. Researchers have hypothesized that bacteria might be washed into shallow lava tubes, or be introduced on plant roots, which often can been seen in the lava tubes (Snider et al., 2009). However, comparisons among soil samples above the lava tubes to determine if there is substantial overlap between the community structures of the two environments was beyond the scope of this study.

The lack of support for our hypothesis that color would be a driving factor of community composition was surprising (Fig. 4). Color is a very visible distinguishing factor in microbial mat appearance, and many microbial mats, especially white mats, look indistinguishable from lava tube to lava tube. There are several possible reasons why color does not appear to be a main driving factor in community composition. First, none of our clone libraries were sampled in depth enough to capture all of the diversity. It may be that with more sequencing, a clearer pattern may emerge. Second, color may also be a result of metabolic processes, and these differences in metabolic processes were not picked up by our analyses of phyla. Finally, color may be due to the presence of other microorganisms that were not included in this study such as *Fungi* or *Archaea*. Soil fungi such as *Fusarium* sp. are known to produce pigments, and have been found to be naturally occurring in other cave systems (Northup et al., 1994). Although there is only minimal evidence of fungi in our SEM studies, it is likely that fungal species are present based on their known presence in caves in general (Nieves-Rivera, 2003).

Actinobacteria have been described from many different cave types, including lava tubes; therefore the presence of *Actinobacteria* in the samples from this study was expected (Groth et al., 2001; Schabereiter-Gurtner et al., 2002; Barton et al., 2004;

Zimmerman et al., 2006; Ikner et al., 2007; Northup et al., 2008; Portillo et al., 2009). A majority of the *Actinobacteria* known fall in the order *Actinobacteridae*, and our results are within this trend and show the considerable novel diversity both within the subclasses *Actinobacteridae* and *Acidimicrobidae* (Zhi et al., 2009). Several clades that were found in only one island indicate that geography may be an important factor in determining composition of bacteria communities. The large number of novel sequences within this group (93% of sequences with <95% similarity) shows the great potential of this ecosystem as an area where new antibiotics can be found (Groth et al., 1999). Several recent studies have described novel cultured *Actinobacteria* species from caves, though none have been from lava tubes (Jurado et al., 2010). Future studies should include culturing specifically for *Actinobacteria* in order to better understand the metabolism and diversity of these organisms.

The results of this study suggest that there are genetically isolated populations of bacteria, refuting Baas-Becking's theory that "everything is everywhere but the environment selects." The analysis of the *Actinobacteria* shows two clades of bacteria from one cave only, Kula Kai, that have less than 96% identity to each other and no close relatives from the Azores. Many studies that have looked for evidence of biogeographic diversity patterns have focused on a single genus (Papke and Ward 2004; Whitaker et al., 2003; Takacs-Vesbach et al., 2008). Although this study did not focus solely on one genus, the overall trends support the Van der Gucht et al. (2007) suggestion that the biogeographical signals will be stronger in rare environments. Furthermore, the low number of OTUs shared by the two locations, 4%, suggests that there may be more factors determining the community structure.

This study provides the first in depth comparison of microbial communities of lava tubes from different locations. These results indicate that geographic location may be important in determining the composition of the bacterial community, and that Hawai‘i seems to be more diverse than the Azores. The results also lend support to conservation of these lava tubes, as novel bacteria were found throughout, especially novel *Actinobacteria*. The study also highlights the need for further exploration of these ecosystems.

Experimental Procedures:

Site Description and Sample Collection: Hawai‘i and the Azores:

Four lava tubes, Kula Kai Caverns, Kaumana, Epperson's, and Bird Park were selected on the Big Island of Hawai'i, in the Pacific Ocean located at 19° 43' N 155° 5' W. The caves represent a variety of abiotic factors, such as temperature, elevation, and yearly precipitation. At each site, entrance elevation, cave temperature and humidity (wet bulb/dry bulb) were recorded, the latter two measured with an IMC Digital Thermometer probe. Average area rainfall was researched and recorded (Table 1). Small samples of wall rock covered with yellow or white microbial mats were collected aseptically from the four Hawaiian Island lava tubes, under a National Park Service collecting permit or permission of land-owners. Samples were selected for collection based on uniformity of color. Samples were covered with sucrose lysis buffer (Giovannoni et al., 1990) to preserve the DNA, and transported to the laboratory where they were stored in a -80° C freezer until DNA extraction.

Terceira is located in the Atlantic Ocean, in the center of the Azores island chain at 38° 44′ N, 27° 17′ W, approximately 1,500 km off the coast of Portugal. White and yellow samples from Terceira were collected from Gruta dos Principiantes, Gruta da Achada, Gruta Branca Opala, and Gruta da Balcões using a similar protocol and covering a similar profile of abotic environmental factors (Table 1).

DNA Extraction, Amplification, and Sequencing:

DNA was extracted and purified using the MoBio PowerSoil™ DNA Isolation Kit using the manufacturer's protocol (MoBio, Carlsbad, CA). Extracted DNA was amplified with universal bacterial primers 46 forward (5'-GCYTAAYACATGCAAGTCG-3') and 1409 reverse (5'-GTGACGGGCRGTGTGTRCAA-3') (Northup et al., 2010). Reactions were carried out in a 25 µl volume with 1X PCR buffer with 1.5mM Mg^{2+}, 0.4µM of each primer, 0.2mM each dNTP, 5µg BSA and 1U AmpliTaq LD (Applied Biosystems, Foster City, CA, USA). Amplification was carried out under the following thermocyling conditions on an Eppendforf Mastercycler 5333 (Eppendorf, Hauppauge, NY): 94°C for 5 min, followed by 31 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.5 min, followed by a final extension of 72°C for 7 min. Amplicons were cleaned and purified using the Qiagen PCR cleanup kit (Qiagen, Germantown, Maryland), and were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA), and sent to Washington University Genome Sequencing Facility for sequencing of 192 clones per sample with primers M13F and M13R.

Table 1. Table of Samples and Abiotic Factors

Sample Name	Cave Name	Location	Color	Elevation (m)	Surface Precipitation (mm)	Surface Land Use	Cave Temp. (°C)	Percent Humidity
BP087	Bird Park	Hawai'i	Yellow	1219	1778	NonPasture	14.3	NA
BP089	Bird Park	Hawai'i	White	1219	1778	NonPasture	14.3	NA
EP097	Epperson's	Hawai'i	White	304	4013	NonPasture	17.7	99
EP912	Epperson's	Hawai'i	Yellow	304	4013	NonPasture	17.7	99
KA613	Kaumana Ranch	Hawai'i	Yellow	610	5003	NonPasture	17.5	99
KA619	Kaumana Ranch	Hawai'i	White	610	5003	NonPasture	17.5	99
KK138	Kula Kai Caverns	Hawai'i	White	415	1016	NonPasture	19	98.1
KK135	Kula Kai Caverns	Hawai'i	Yellow	415	1016	NonPasture	19	98.1
AS252	Gruta da Achada	Azores	Yellow	330	1635	NonPasture	14.9	98.5
AS253	Gruta da Achada	Azores	White	330	1635	NonPasture	14.9	98.5
BL128	Gruta da Balcões	Azores	White	422	1967	Pasture	16	99.5
GBL12	Gruta da Balcões	Azores	Yellow	422	1967	Pasture	16	99.5
BO654	Gruta da Branca Opala	Azores	White	255	1400	NonPasture	15	95
GBO52	Gruta da Branca Opala	Azores	Yellow	255	1400	NonPasture	15	95
GP276	Gruta dos Principiantes	Azores	White	346	1728	Pasture	15.4	98.5
GP283	Gruta dos Principiantes	Azores	Yellow	346	1728	Pasture	15.4	98.5

Molecular Phylogenetics:

Sequences were edited and assembled with Sequencher 4.8. (Gene Codes, Ann Arbor, Michigan). Orientation was checked with OrientationChecker (www.cardiff.ac.uk/biosi/research/biosoft). Chimeras were detected using the Mallard/Pintail software (http://www.bioinformatics-toolkit.org). Initial alignment was completed with Greengenes (greengenes.lbl.gov; DeSantis et al., 2006) and manually corrected using BioEdit editor (www.mbio.ncsu.edu/BioEdit/bioedit.html), guided by 16S rRNA secondary structure considerations. Sequences were then classified at the phylum level using the Ribosomal Database Project classifier (RDP) (rdp.cme.msu.edu; Maidak et al., 2001). Alignments were then imported into ARB using a reference tree of either ~9000 full length sequences from RDP or 236,469 full-length sequences from Greengenes (greengenes.lbl.gov/Downloads/ Sequence_Data; Hugenholtz, 2002; Ludwig et al., 2004). Rarefaction curves and nonparametric richness estimates were generated in *mothur* to evaluate sampling efforts (Schloss et al., 2009; Chao, 1984). Sequences were compared with the GenBank database using basic local alignment search tool (BLAST) (Altschul et al., 1990). Samples were also compared using UniFrac to determine if geography and color were important factors in determining community profiles using an unweighted Principal Component Analysis (Lozupone and Knight, 2005). Parsimony analysis was performed on a subset of 89 OTUs that were identified as *Actinobacteria* using PAUP (version 4.0b10, distributed by Sinauer; paup.csit.fsu.edu/; Swofford, 2000) and bootstrap analyses were conducted with 1000 replicates using PAUP.

Scanning Electron Microscopy:

Samples were examined on a JEOL 5800 SEM equipped with an energy dispersive x-ray analyzer (EDX). Rock chips with microbial mats adhered were mounted directly on SEM sample stubs in the field and coated with Au-Pd metal for imaging in the laboratory.

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Chapter 3

Diversity of Ammonia Oxidation (*amoA*) and Nitrogen Fixation (*nifH*) Genes in Lava Tubes of Terceira, Azores, Portugal

Introduction

The cycling of nitrogen provides energy and essential forms of nitrogen for many types of microorganisms. Organisms responsible for nitrogen transformations have been well studied in soil and aquatic habitats, yet new discoveries are still being made, such as the discovery of *Archaea* that perform ammonia oxidation (Venter *et al*., 2004; Treusch *et al*., 2005; McCarthy *et al*., 2007; Collins *et al.*, 2008; Prosser and Nicol, 2008; Ross *et al*., 2009; Sooksa-nguan *et al*., 2009). Certain nitrogen cycling processes can provide energy as well as nutrients for microbial communities that grow in oligotrophic environments, such as caves. For closed or semi-closed environments, nitrogen fixation by *Bacteria* and *Archaea* can be the main source of bioavailable nitrogen for plants and microorganisms, while ammonia produced as a product of organic matter mineralization can be a source of energy for chemolithotropic organisms. Studies of lithotrophic ammonia oxidation in subsurface environments are rare (Simon and Benfield, 2002; Spear *et al*., 2007; Chen *et al*., 2009). More generally, there has been little study of nitrogen cycling or the diversity of the organisms that carry out nitrogen transformations in the subsurface environments.

We investigated the diversity of genes that encode the enzymes that mediate nitrogen transformation processes in microbial mats collected in lava tubes of Terceira, Azores, Portugal. Nitrogenase, which is the enzyme responsible for the fixation of nitrogen, is partially encoded by the gene *nifH*. Nitrogen fixation is an energetically expensive process that is done anaerobically. It has not been directly measured in oligotrophic cave environments, but putative nitrogen fixing microbial taxa have been found in carbonate caves (Barton *et al*., 2004; Dichosa, 2008).

The limiting step in nitrification, the transformation of ammonia to nitrate, is the conversion of NH_4^+ to NH_2OH. This process is controlled by the enzyme ammonia mono-oxygenase, which is partly encoded by the highly conserved gene *amoA*. Ammonia is not stable in oxic environments and is quickly oxidized by ammonia oxidizing bacteria (AOB) and archaea; however, moderate levels of ammonia have been found in ferromanganese deposits in Spider and Lechuguilla Caves in New Mexico, giving evidence of the availability of substrates for ammonia oxidation (Northup *et al*., 2003). The authors hypothesize that the ammonium is bound to the clay particles in the ferromanganese deposits and hence is biological unavailable. However, the presence of ammonia may be an indication of nitrogen fixation, whose end product is ammonia.

Analyses of nitrogen cycling have not been conducted in subterranean habitats even though nitrogen limitation is thought to be a major constraint on biological productivity. Simon and Benfield (2002) looked at nitrogen levels in a karst cave stream, and found that nitrogen was not as limiting as carbon in that aquatic environment. However, there is little data regarding nitrogen availability in non-aquatic cave environments. Two studies have looked for and found the presence of ammonia oxidizing genes in the subsurface. Spear *et al*. (2007) found archaeal *amoA* in a mine adit and Chen *et al*. (2009) found bacterial *amoA* in a carbonate cave. Neither study investigated if the genes were being expressed in the subsurface. No studies to date have looked specifically for the presence of nitrogen fixation genes in cave environments.

Lava tubes, volcanically formed caves, are generally more shallow subterranean environments than limestone caves. Their shallowness, combined with the tendency for lava tubes to have cracks in the overlying volcanic rocks, results in more connectivity to

the surface compared to many limestone caves (Howarth, 1996). The extensive colorful microbial mats that cover the walls and ceilings of lava tubes have long been described, but the composition and diversity of the mats is just being explored with culture-independent techniques (Staley and Crawford, 1975; Stoner and Howarth, 1981; Northup *et al*., 2008; Garcia *et al*., 2009; Moya *et al*., 2009; Snider *et al*., 2009). 16S rDNA bacterial diversity of lava tubes from these studies suggests that groups of bacteria capable of nitrogen fixation or ammonia oxidation are present in lava tubes (Garcia *et al*., 2009; Moya *et al*., 2009; Snider *et al*., 2009). Studies of other types of basaltic environments have shown that lava is deficient in both carbon and nitrogen, and bacteria that can fix nitrogen play an important role in the establishment of other bacterial communities in these environments (King, 2003; Mason *et al*., 2009).

We investigated nitrogen fixation and ammonia oxidation in 11 lava tubes on Terceira Island in the Azores, Portugal. Terceira has lava tubes across the island, some underneath extensive cow pastures and some underneath forested areas of both native and exotic plants. The soils overlying the lava tubes are classified as andisols (Madeira *et* al., 2007). Because of the high precipitation rate and high hydrologic connectivity between the lava tubes and the surface, we hypothesized that nutrient inputs to lava tubes would vary with land use and elevation. In response to these differences, we predicted that the diversity of *nifH* and *amoA* genes in the microbial mats of lava tubes would show a complementary pattern of variation.

Materials and Methods

Sample Site Description:

Terceira is located in the Atlantic Ocean, in the center of the Azorean island chain at 38° 44′ N, 27° 17′ W, approximately 1,500 km off the coast of Portugal. Eleven lava tubes were selected to represent a range of elevation, annual precipitation, and surface land use (Figs. 1, 2; Table 1).

Sample collection:

Samples of microbial mats were collected using aseptic methods in February and June 2008. Yellow, white and tan samples were collected from each cave, as well as microbial mats of unique colors, such as pink, grey and black. Samples were selected for collection based on uniformity of color. Samples were covered with sucrose lysis buffer to preserve the DNA (Giovannoni *et al*., 1990) and transported to the lab where they were stored at -80°C until DNA was extracted. Water for nutrient analyses and soil samples for carbon/nitrogen analyses were collected in June 2008 and July 2009. Samples were kept at 4°C until analysis. Temperature and humidity data (wet bulb/dry bulb) was collected throughout the cave with an IMC Digital Thermometer probe. This method does not allow for accurate humidity measurements above 95%.

Water and soil chemistry:

Dissolved organic carbon (DOC) in infiltrating water was collected and passed through a 0.45μm filter and preserved at pH 2 using HCL on site, as described in Simon *et al*. (2007). Organic and inorganic carbon water samples were analyzed using the persulfate digestion method as described in Clescerl *et al*. (1999) on a Shimadzu TOC-5050A instrument (Shimadzu Corporation, Kyoto, Japan). Amounts of sulfate, nitrate,

and phosphate were analyzed using a Dionex Ion Chromatograph DX-100 (Dionex, Sunnyvale, CA) as described in Pfaff *et al*. (1997). The amount of ammonia in the water samples was analyzed using a Technicon AutoAnalyzer II (Technicon, Tarrytown, NY).

Soil samples were collected from the floor near the entrance of each lava tube and deeper within the interior of the lava tube. Percent nitrogen and percent carbon in soil were determined by high temperature combustion, the resulting gases were eluted on a gas chromatography column and detected by thermal conductivity and integrated to yield carbon and nitrogen content. Analyses were performed on a ThermoQuest CE Instruments NC2100 Elemental Analyzer (ThermoQuest Italia S.p.A., Rodano, Italy) (Pella, 1990a; Pella, 1990b.) The results from soil samples collected from within one lava tube were then averaged.

Molecular Methods:

DNA was extracted and purified from 55 samples from the 11 lava tubes on Terceira, using the MoBio PowerSoil™ DNA Isolation Kit using the manufacturer's protocol, with the modification of bead beating instead of vortexing to break open cells (MoBio, Carlsbad, CA). Extractions with no sample added were performed as negative controls. Samples were then screened for the two functional genes, *amoA* and *nifH* using polymerase chain reaction (PCR). For *amoA*, the primers amoA1F and amoA2R were used under conditions described in Table 2. Reactions were carried out in a 25 µL reaction mixture containing 1X PCR buffer with 2mM Mg^{2+}, 0.2mM each dNTP, 0.4µM of each primer, 5µg BSA and 0.75 U of TaKaRa Ex Taq (TaKaRa, Shiga, Japan).

Table 1: Abiotic factors associated with each cave. Land use describes how the land above the lava tube is used by humans, or the nature of the vegetation above the lava tube where no human use is present.

Cave Name	Elevation (m)	Surface Precipitation (mm)	Temperature (°C)	Relative Humidity (%)	Land Use
Algar do Carvão	585	2303	11.3	N/A	Forested
Gruta da Branca Opala	255	1400	15.0	95.2	Forested
Gruta da Madre de Deus	59	1050	14.6	100.0	Forested
Gruta da Achada	330	1635	14.9	98.5	Forested
Gruta das Agulhas	1	1015	22.1	N/A	Sea/Urban
Gruta do Natal	551	2253	15.7	N/A	Pasture
Gruta da Terra Mole	387	1809	14.9	99.8	Pasture
Gruta da Malha	507	2135	15.5	99.0	Pasture
Gruta dos Buracos	475	2034	15.7	99.4	Pasture
Gruta dos Principiantes	346	1728	15.4	98.5	Pasture
Gruta da Balcões	422	1967	16.0	99.5	Forested

Figure 1. Map of Terceira Island, with the location of the lava tubes.

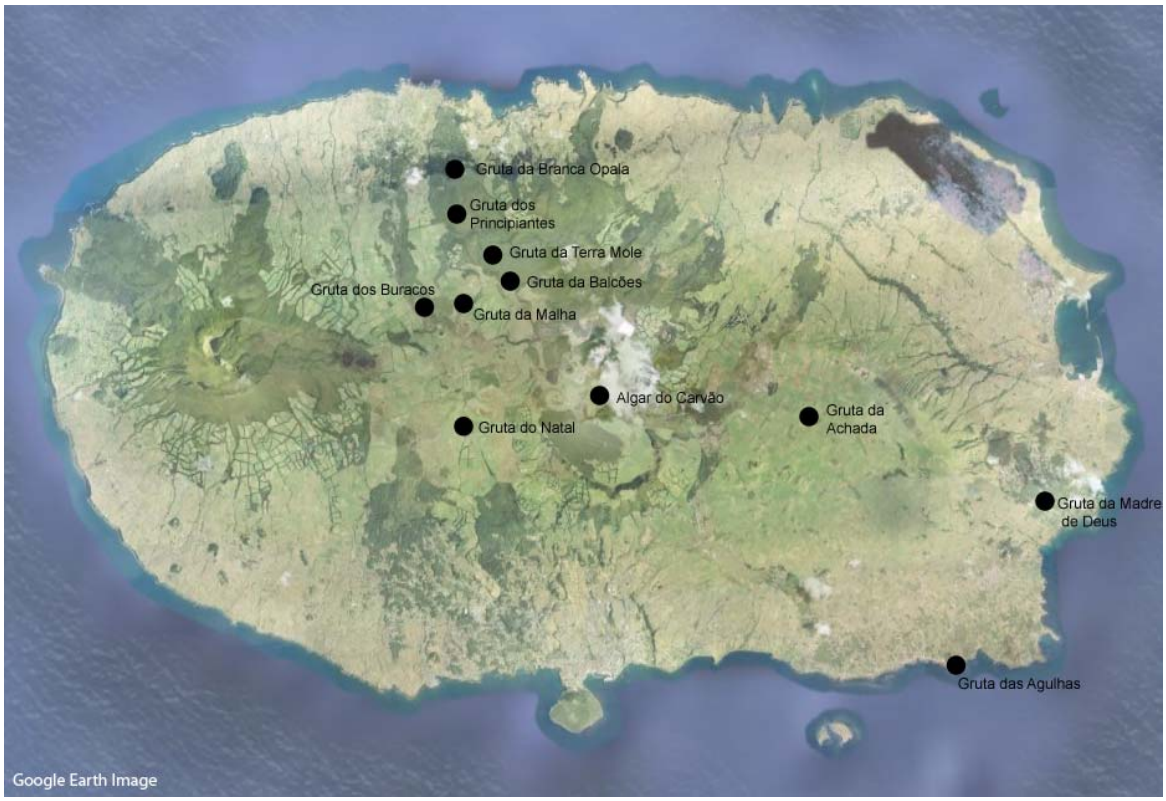


Figure 2. Images showing different land use practices, and bacterial mats within the lava tubes of Terceira. A) Above Gruta da Madre de Deus. B) Bacterial mat within Gruta da Madre de Deus. C) Above Gruta da Balcões. D) Bacterial mat within Gruta da Balcões.

Table 2. Primers and thermocycling conditions

Primer	Sequence 5' to 3'	Length of amplicon	Amplification conditions	Reference
amoA1F	GGGGTTTCTACTGGTGGT	490	5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C, 1min at 72°C, with a final extension at 72°C for 10 minutes	Rotthauwe *et al.* 1997
amoA2R	CCTCKGSAAAGCCTTCTTC			Rotthauwe *et al.* 1997
amoA-1F-GC	CGCCCGCCGCGCCCCGC GCCCGGCCCGCCGCCC CCGCCCCGGGGTTTC TACTGGTGGT			Shen *et al.*2008
FGPH19	TACGGCAARGGTGGNATHG	432	2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C, final extension of 5 min at 72°C	Simonet *et al.* 1991
PolR	ATSGCCATCATYTCRCCGGA			Poly *et al.* 2001
PolF	TGCGAYCCSARGCBGACTC	321	2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C, final extension of 5 min at 72°C	Poly *et al.* 2001
AQER	GACGATGTAGATYTCCTG			Poly *et al.* 2001
PolF GC	CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACG GGGGGTGCGAYCCSAR GCBGACTC			Demba-Diallo et al.2004

For *nifH*, a nested PCR was required with primers FGPH19 and PolR used in the first round PCR and AQER and PolF used in the second round (Table 2). Reactions were carried out in a 50μL reaction mixture containing 1X PCR buffer with 1.5mM Mg^{2+}, 0.8μM of each primer, 0.2mM each dNTP, 5μg BSA and 1U AmpliTaq LD (Applied Biosystems, Foster City, CA, USA). Three microliters of PCR product from round one was used in the second round PCR, with the same conditions as above, except the primer concentration was decreased to 0.4 μM.

Denaturing Gradient Gel Electrophoresis:

For any sample that was positive for the gene, a denaturing gradient gel electrophoresis (DGGE) was performed. The samples were amplified using the same primer sets, with a GC clamp attached to the 5' end of the forward primer (Table 2). Samples were run on an 8% (w/v) bis-acrylamide gel with a urea-formamide gradient of 40% to 70% (w/v) for 16 hrs at 110 V in 17 L of 60°C 1x TAE buffer on a CBS DGGE-1001 (CBS Scientific, Del Mar, CA).

Gels were stained in 1X SYBRGold (Molecular Probes, Eugene, OR), and imaged on a Sygene InGenius Bio Imager (Sygene, Frederick, MD). Individual bands were then excised, reamplifed under the same conditions as the initial amplification, with only the second primer set used for *nifH*, and cleaned using the MoBio's UltraClean ™ PCR-Clean-up ™ (MoBio, Carlsbad, CA). Bands that were successfully reamplifed were then sequenced with Big Dye Terminator v1.1 using 5 ul of PCR product (Applied Biosystems, Foster City, CA), and sequenced on an ABI 3130 sequencing machine (Applied Biosystems, Foster City, CA).

Sequences were edited for quality using Sequencher 4.9 (Gene Codes, Ann Arbor, MI), and then aligned using MUSCLE (www.ebi.ac.uk/Tools/muscle; Edgar, 2004). Sequences from the same sample with >97% similarity were defined as the same OTU using mothur (Schloss et al., 2009). Community analyses were also preformed in mothur. Parsimony analyses were performed using PAUP (version 4.0b10, distributed by Sinauer; http://paup.csit.fsu.edu/) with a bootstrap analysis conducted on 1000 re-sampled datasets.

Results

Water and soil chemistry:

The water and soil chemistry results are summarized in Table 3. Samples from under forested land were not significantly different from those from under pasture-land in regards to the amount of organic carbon (water p= 0.5, soil p=0.48) and nitrogen (water p=0.44, soil p=0.5) in material entering the lava tube.

Ammonia oxidation:

A total of 14 of the 55 samples tested were positive for the *amoA* gene, from six of the 11 lava tubes, three under forested land, one under sea/urban land and two under pasture land. Most samples had approximately 6-10 bands per lane, although some lanes such as GBO1-1 from Gruta Branca Opala had considerably more, with over 15 bands. Seventy-eight bands were excised and sequenced from the 14 samples (Fig. 3, Table 4). Using a 97% similarity cut-off, 15 unique OTUs were designated. Ten OTUs were found exclusively in forested lava tubes, one exclusively in a pasture lava tube, three were shared between pasture lava tubes and forested lava tubes, and one was shared between

Table 3. Water and Soil Chemistry

		Water							Soil		
Cave	**Abbreviation**	**PO_4^-**	**SO_4**	**TOC**	**IC**	**NO_3^-**	**NH_4^+**	**TN**	**% N**	**%C**	**Land Use**
Algar do Carvão	AC	0.0374	4.10	0.86	4.14	0.08	0.060	0.31	0.1414	3.012	Forested
Gruta da Branca Opala	GBO	0.0312	1.42	1.24	0.16	2.12	0.040	2.46	0.8104	8.494	Forested
Gruta da Madre de Deus	GMD	0.0696	5.55	5.40	2.37	0.86	0.020	1.38	0.1480	2.056	Forested
Gruta da Achada	GAS	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Sea/Urban
Gruta das Agulhas	GA	0.0035	37.29	1.10	2.35	6.92	0.090	10.20	0.0930	1.397	Pasture
Gruta do Natal	GN	0.0350	6.36	2.32	4.82	0.75	0.080	1.86	0.1873	1.998	Pasture
Gruta da Terra Mole	GTM	0.0199	5.43	1.19	0.09	1.03	0.020	1.26	0.7076	10.151	Pasture
Gruta da Malha	GML	0.0027	3.54	1.45	2.11	3.75	0.040	5.96	0.1691	3.864	Pasture
Gruta dos Buracos	GB	0.0029	1.87	8.10	2.80	0.28	0.030	1.08	0.2342	4.559	Pasture
Gruta dos Principiantes	GP	0.0013	1.94	0.78	1.85	1.78	0.030	2.20	0.3047	4.357	Pasture
Gruta da Balcões	GBL	0.0030	5.19	1.17	2.29	0.46	0.030	1.17	0.6622	7.871	Pasture

forested lava tubes and the sea/urban lava tube (Fig. 5A). Thirteen of the OTUs were similar to known species of *Nitrosospira*, and the other two OTUs were similar to *Nitrosomonas* sequences (Fig. 6). Several of the closest relatives to sequences recovered in this study were uncultured soil bacterium clones. *Nitrosospira*-like sequences were seen in all samples except the one from Gruta das Agulhas, the lava tube that opens into the sea. There were no OTUs shared by all samples.

***Nitrogen fixation*:**

A total of 30 of the 55 samples screened were positive for the *nifH* gene, from all 11 lava tubes surveyed. Most samples had approximately 5-7 bands, but GBO54 and GB1927 had considerably more. One hundred forty-six bands were sequenced, and were classified into 22 unique OTUs using a 97% similarity cut-off (Fig. 4, Table 5). Nine of the OTUs were unique to the forested lava tubes, seven unique to the pasture lava tube, and one OTU was unique to the sea/urban lava tube (Fig. 5B). There were two OTUs shared by the forested and pasture lava tubes, one shared by the pasture and sea/urban lava tube, one shared by the forested and sea/urban lava tube, and one OTU shared by all three land use categories. Sixty-one of the 146 bands (41.7%) of the sequences recovered were identified as *Klebsiella pneumoniae*-like sequences. *K. pneumoniae*-like sequences were found in 13 samples from eight different lava tubes. Twenty-two of the 146 sequences (15.1%) were identified as *Zoogloea oryzae*-like. Nitrogen fixing bacteria from three phyla of bacteria were found: *Actinobacteria* (2 sequences), *Firmicutes* (12 sequences), and four *Proteobacteria* classes, *alpha* (25 sequences), *beta* (38 sequences), *delta* (2 sequences) and *gamma* (67 sequences) (Fig. 7). There were no OTUs shared by all samples.

Table 4. *amoA* DGGE Band Identification. Putative ID shows genus classifications.

DGGE Sequence	Genbank match	Putative ID	OTU number	OTU name
A-AC30_3A	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-AC30_3B	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-AC30_3C	AJ538109	*Nitrosospira*	2	A-AC30_3C
A-AC30_7A	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-AC30_7B	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-AC30_7C	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-GA21_10A	EU244515	*Nitrosomonas*	3	A-GA21_10A
A-GA21_10B	EU244515	*Nitrosomonas*	3	A-GA21_10A
A-GA21_10C	EU244515	*Nitrosomonas*	3	A-GA21_10A
A-GAS25_1A	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-GAS25_1B	DQ480773	*Nitrosospira*	1	A-AC30_3A
A-GAS25_2A	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_2B	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_2C	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_2D	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_2E	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4A	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4B	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4C	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4D	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4J	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4K	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_4L	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_5A	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_5B	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_5C	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_5D	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_5G	EU244515	*Nitrosomonas*	3	A-GA21_10A
A-GAS25_5H	DQ480827	*Nitrosospira*	4	A-GAS25_2A
A-GAS25_5I	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GB01_1D	DQ480773	*Nitrosospira*	1	A-AC30_3A
J-GB01_3C	DQ480773	*Nitrosospira*	1	A-AC30_3A
J-GB19_27A	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GB19_27B	DQ480827	*Nitrosospira*	4	A-GAS25_2A

DGGE Sequence	Genbank match	Putative Genus	OTU number	OTU name
J-GB19_27C	DQ480773	*Nitrosospira*	1	A-AC30_3A
J-GB19_27D	AY786052	*Nitrosospira*	8	J-GB19_27D
J-GB19_27E	AY786052	*Nitrosospira*	8	J-GB19_27D
J-GBO1_1B	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GBO1_1C	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GBO1_1E	DQ480773	*Nitrosospira*	1	A-AC30_3A
J-GBO1_1G	DQ480778	*Nitrosospira*	9	J-GBO1_1G
J-GBO1_1H	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GBO1_1I	DQ480772	*Nitrosospira*	5	J-GBO1_1I
J-GBO1_1J	DQ480772	*Nitrosospira*	5	J-GBO1_1I
J-GBO1_1L	DQ480773	*Nitrosospira*	6	J-GBO1_1L
J-GBO1_3A	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GBO2654B	DQ480827	*Nitrosospira*	4	A-GAS25_2A
J-GBO2654D	EF544031	*Nitrosospira*	7	J-GBO2654D
J-GBO2654E	EF544031	*Nitrosospira*	7	J-GBO2654D
J-GBO2654F	EF544031	*Nitrosospira*	7	J-GBO2654D
J-GBO2654G	X90821	*Nitrosospira*	10	J-GBO2654G
J-GBO2654H	EF544031	*Nitrosospira*	7	J-GBO2654D
J-GTM18_10A	DQ480773	*Nitrosospira*	1	A-AC30_3A
J-GTM18_10B	DQ480773	*Nitrosospira*	1	A-AC30_3A
J-GTM2_1A	AJ538109	*Nitrosospira*	2	A-AC30_3C
J-GTM2_3A	AJ538109	*Nitrosospira*	2	A-AC30_3C
S-AC30_7A	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-AC30_7B	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-AC30_7C	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-AC30_7D	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-AC30_7E	DQ480786	*Nitrosomonas*	11	S-AC30_7E
S-AC30_7F	DQ480786	*Nitrosomonas*	11	S-AC30_7E
S-GBO1_1A	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-GBO1_1B	AF239881	*Nitrosospira*	12	S-GBO1_1B
S-GBO1_1C	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-GBO1_1E	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-GBO1_1F	DQ480827	*Nitrosospira*	4	A-GAS25_2A
S-GBO1_1G	DQ480827	*Nitrosospira*	4	A-GAS25_2A
S-GBO1_1H	DQ480773	*Nitrosospira*	6	J-GBO1_1L

DGGE Sequence	Genbank match	Putative Genus	OTU number	OTU name
S-GBO1_1I	DQ480773	*Nitrosospira*	1	A-AC30_3A
S-GBO2654B	DQ480827	*Nitrosospira*	4	A-GAS25_2A
S-GBO2654D	DQ480827	*Nitrosospira*	4	A-GAS25_2A
S-GBO2654E	DQ480827	*Nitrosospira*	13	S-GBO2654E
S-GBO2654F	X90821	*Nitrosospira*	14	S-GBO2654F
S-GBO2654G	X90821	*Nitrosospira*	14	S-GBO2654F
S-GBO2654H	DQ480827	*Nitrosospira*	4	A-GAS25_2A
S-GBO2654I	AF239881	*Nitrosospira*	12	S-GBO1_1B

Table 5. *nifH* DGGE Band Identification. Putative ID shows phylum and more derived classifications if available in parentheses

DGGE Sequence	Genbank match	Putative ID	OTU Number	OTU name
16SGB19261	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGB19262	DQ821976	*Betaproteobacteria*	22	26AGA2196
16SGB19263	DQ821976	*Betaproteobacteria*	22	26AGA2196
16SGB19264	DQ821976	*Betaproteobacteria*	22	26AGA2196
16SGB192710	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB192711	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB192713	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19272	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19273	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19274	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19275	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19277	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19278	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGB19279	AY196394	*Alphaproteobacteria*	16	16SGB192710
16SGBL1281	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1282	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1283	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1284	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1285	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1286	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1287	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1288	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
16SGBL1289	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
23SGAS34	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
23SGAS37	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
23SGBO171	EU978412	*Alphaproteobacteria*	18	23SGBO171
23SGBO172	EU978412	*Alphaproteobacteria*	18	23SGBO171
23SGBO174	DQ821727	*Gammaprotoebacteria (Klebsiella)*	9	26AGBO522
23SGBO175	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
23SGBO541	AM746599	*Deltaproteobacteria*	3	23SGBO541
23SGBO5410	AY373369	*Firmicutes (Bacillus)*	5	23SGBO5410
23SGBO5411	AY373370	*Firmicutes (Bacillus)*	5	23SGBO5410
23SGBO5413	AY373371	*Firmicutes (Bacillus)*	5	23SGBO5410
23SGBO5414	AY373372	*Firmicutes (Bacillus)*	5	23SGBO5410

DGGE Sequence	Genbank match	Putative ID	OTU Number	OTU name
23SGBO5415	AY373373	*Firmicutes (Bacillus)*	5	23SGBO5410
23SGBO543	EF521086	*Alphaproteobacteria (Methylocystis)*	13	23SGBO543
23SGBO547	AY373374	*Firmicutes (Bacillus)*	5	23SGBO5410
23SGBO548	AY373375	*Firmicutes (Bacillus)*	5	23SGBO5410
23SGBO549	AY373376	*Firmicutes (Bacillus)*	5	23SGBO5410
26AAC3031	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC30310	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3032	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3033	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3034	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3035	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3036	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3037	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3038	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AAC3071	AY684107	*Gammaproteobacteria*	12	26AAC3071
26AAC3072	AY684107	*Gammaproteobacteria*	12	26AAC3071
26AAC3073	AY684107	*Gammaproteobacteria*	12	26AAC3071
26AGA2121	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2122	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2123	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2124	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2125	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2126	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2127	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2141	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2142	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2143	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2144	DQ821727	*Gammaprotoebacteria (Klebsiella)*	8	26AGA2144
26AGA2191	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2192	DQ821976	*Betaproteobacteria*	22	26AGA2196
26AGA2193	DQ821976	*Betaproteobacteria*	22	26AGA2196
26AGA2194	DQ821976	*Betaproteobacteria*	22	26AGA2196
26AGA2195	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGA2196	DQ821976	*Betaproteobacteria*	22	26AGA2196
26AGAS21	AY351671	*Actinobacteria (Frankia)*	2	26AGAS21

DGGE Sequence	Genbank match	Putative ID	OTU Number	OTU name
26AGAS22	AY351671	*Actinobacteria (Frankia)*	2	26AGAS21
26AGAS41	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGAS42	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGAS43	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGAS44	GQ464080	*Alphaproteobacteria*	21	26AGAS44
26AGAS45	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGAS46	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGAS47	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGBO111	AB373746	*Alphaproteobacteria*	20	26AGBO111
26AGBO112	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGBO114	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGBO115	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGBO171	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
26AGBO172	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
26AGBO173	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
26AGBO521	DQ821727	*Gammaprotoebacteria (Klebsiella)*	6	26AGBO521
26AGBO522	DQ821727	*Gammaprotoebacteria (Klebsiella)*	9	26AGBO522
26AGBO523	DQ821727	*Gammaprotoebacteria (Klebsiella)*	9	26AGBO522
26AGBO525	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD11	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD12	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD13	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD14	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD15	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD16	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD17	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD18	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD31	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD32	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD33	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD34	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
26AGMD35	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGA2111	AY684107	*Gammaproteobacteria*	12	26AAC3071
2SGA2112	AY684107	*Gammaproteobacteria*	12	26AAC3071

DGGE Sequence	Genbank match	Putative ID	OTU Number	OTU name
2SGA2113	AY684107	*Gammaproteobacteria*	12	26AAC3071
2SGBL102	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGBL104	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGBL105	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGBL106	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGBO541	AY223975	*Deltaproteobacteria*	1	2SGBO541
2SGBO543	AY373377	*Firmicutes (Bacillus)*	5	23SGBO5410
2SGBO544	AY373378	*Firmicutes (Bacillus)*	5	23SGBO5410
2SGBO545	AY373379	*Firmicutes (Bacillus)*	5	23SGBO5410
2SGBO546	AY373380	*Firmicutes (Bacillus)*	5	23SGBO5410
2SGML1791	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGML1792	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
2SGN411	AY196378	*Betaproteobacteria*	10	2SGN411
31AGBL111	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
31AGBL112	DQ776549	*Betaprotoebacteria (Burkholderiales)*	15	26AGBO171
31AGML111	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGML112	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGML114	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGN451	AY196378	*Betaproteobacteria*	10	2SGN411
31AGN452	AY196378	*Betaproteobacteria*	10	2SGN411
31AGN453	AY196378	*Betaproteobacteria*	10	2SGN411
31AGN455	AY196378	*Betaproteobacteria*	10	2SGN411
31AGN456	AY196378	*Betaproteobacteria*	10	2SGN411
31AGN457	AY196378	*Betaproteobacteria*	11	31AGN457
31AGP2721	DQ098219	*Alphaproteobacteria*	17	31AGP2721
31AGP27210	AF331977	*Betaproteobacteria*	4	31AGP27210
31AGP2723	GQ464080	*Alphaproteobacteria*	19	31AGP2723
31AGP2724	GQ464080	*Alphaproteobacteria*	21	26AGAS44
31AGP2725	GQ464080	*Alphaproteobacteria*	19	31AGP2723
31AGP2729	DQ821727	*Gammaprotoebacteria (Klebsiella)*	7	26AGA2195
31AGTM101	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM102	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM103	DQ821976	*Betaproteobacteria*	22	26AGA2196

DGGE Sequence	Genbank match	Putative ID	OTU Number	OTU name
31AGTM1831	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM1832	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM1833	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM211	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM212	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM213	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM214	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM231	DQ779147	*Betaprotoebacteria (Burkholderiales)*	14	31AGTM231
31AGTM232	DQ821976	*Betaproteobacteria*	22	26AGA2196
31AGTM233	DQ821976	*Betaproteobacteria*	22	26AGA2196

Figure 3. DGGE image of *amoA* gels. Sample names are along the top while individual bands sequenced are labeled within each gel. S, A, and J designate different gels runs.

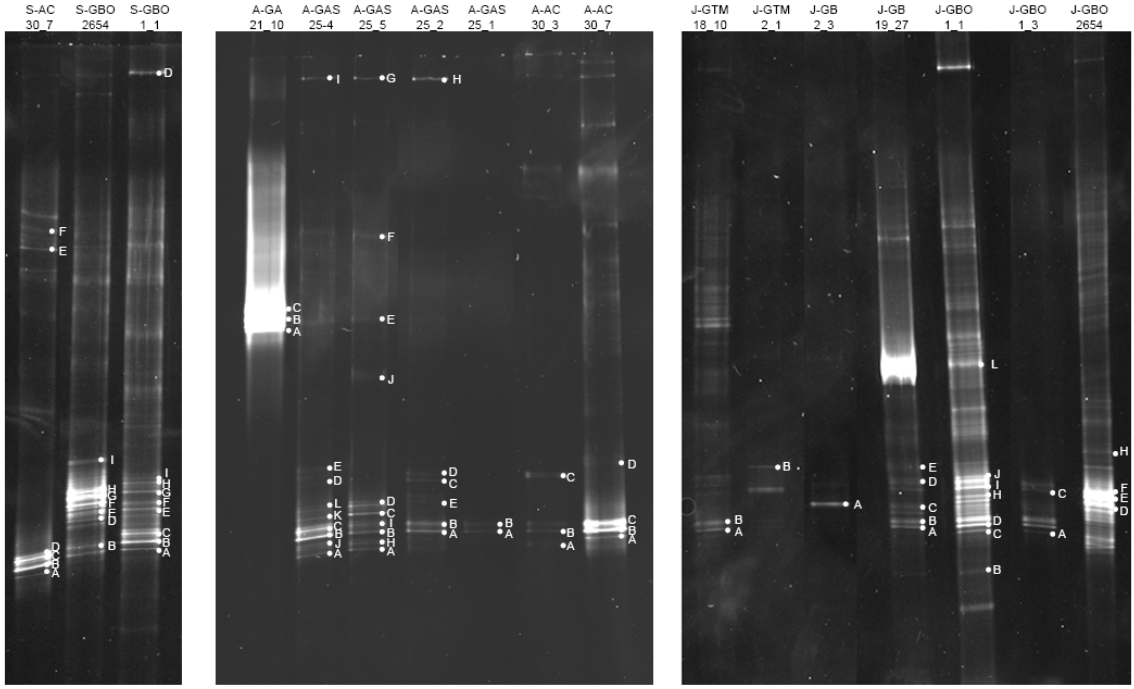


Figure 4. DGGE image of *nifH* gels. Sample names are along the top while individual bands sequenced are labeled within each gel. 26A, 31A, 2S, 16S and 23S designate different gel runs.

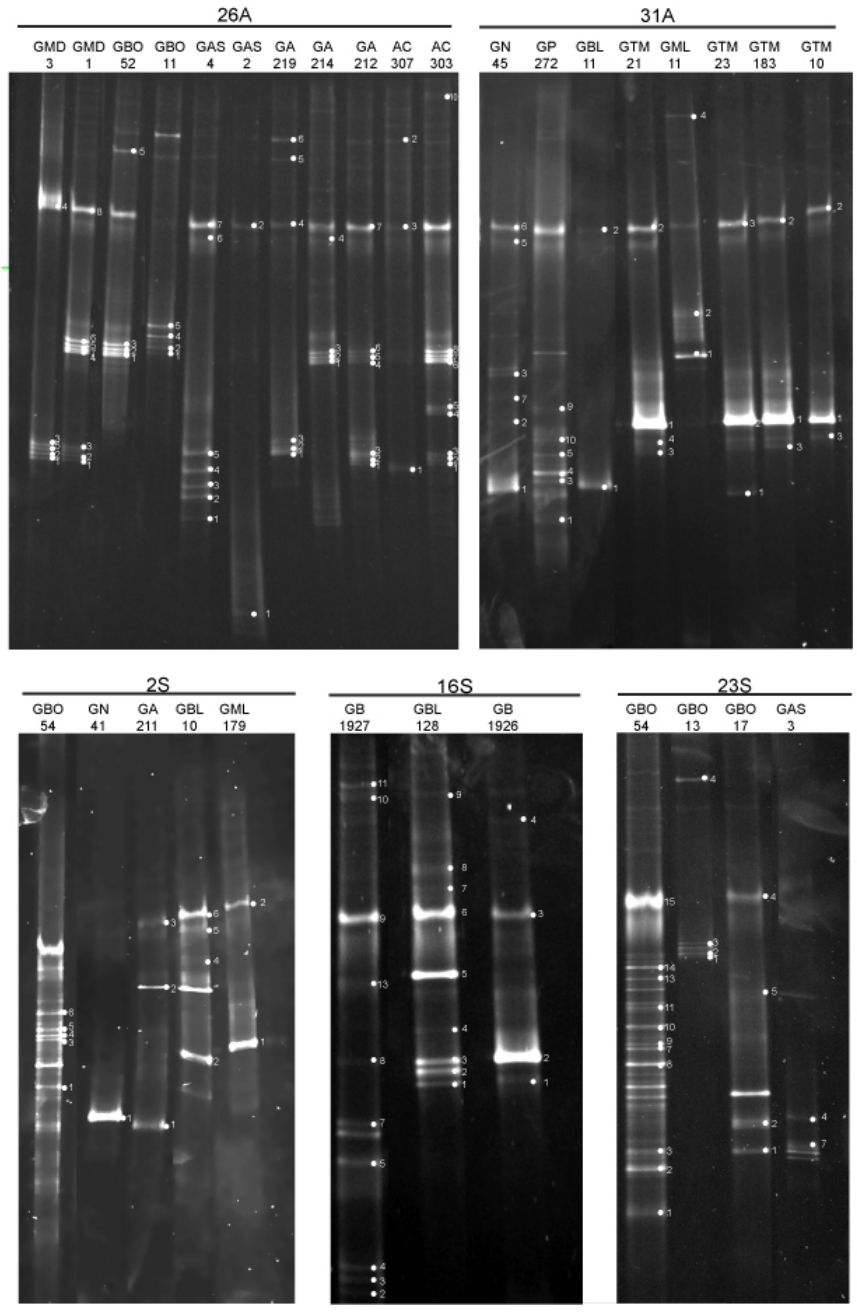


Figure 5. Venn diagrams showing unique and overlapping OTUs for (A) *amoA* and (B) *nifH* DGGE results, based on the land use above the lava tube.

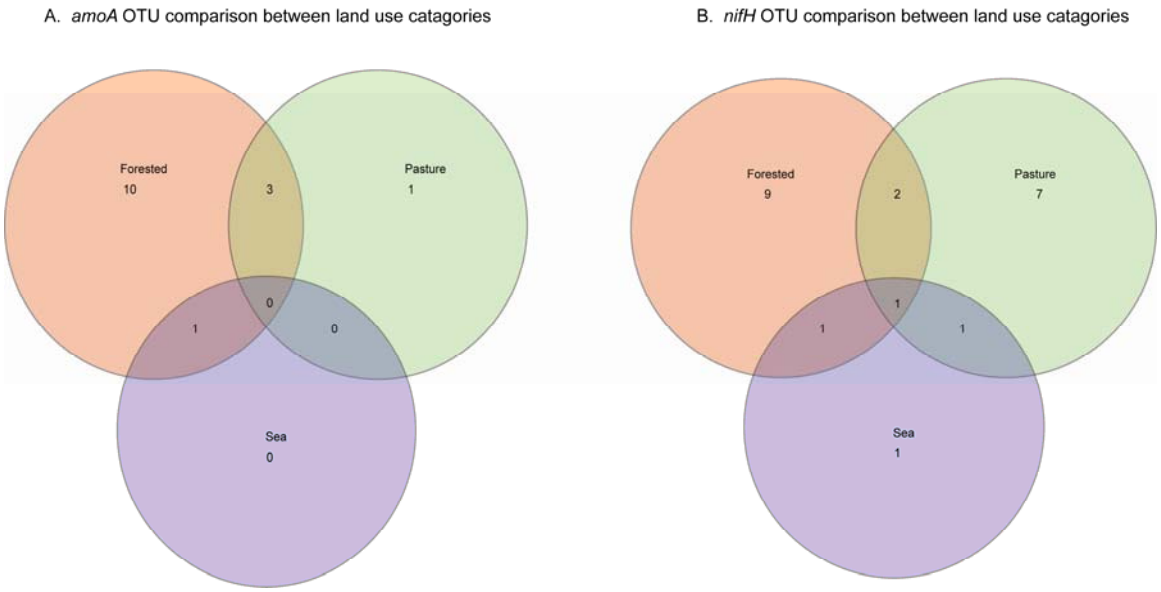


Figure 6. One of the most parsimonious trees of *amoA* OTUs. Bootstrap values for 1000 replicates are shown. Number in parentheses indicates how many sequences fell within the OTU.

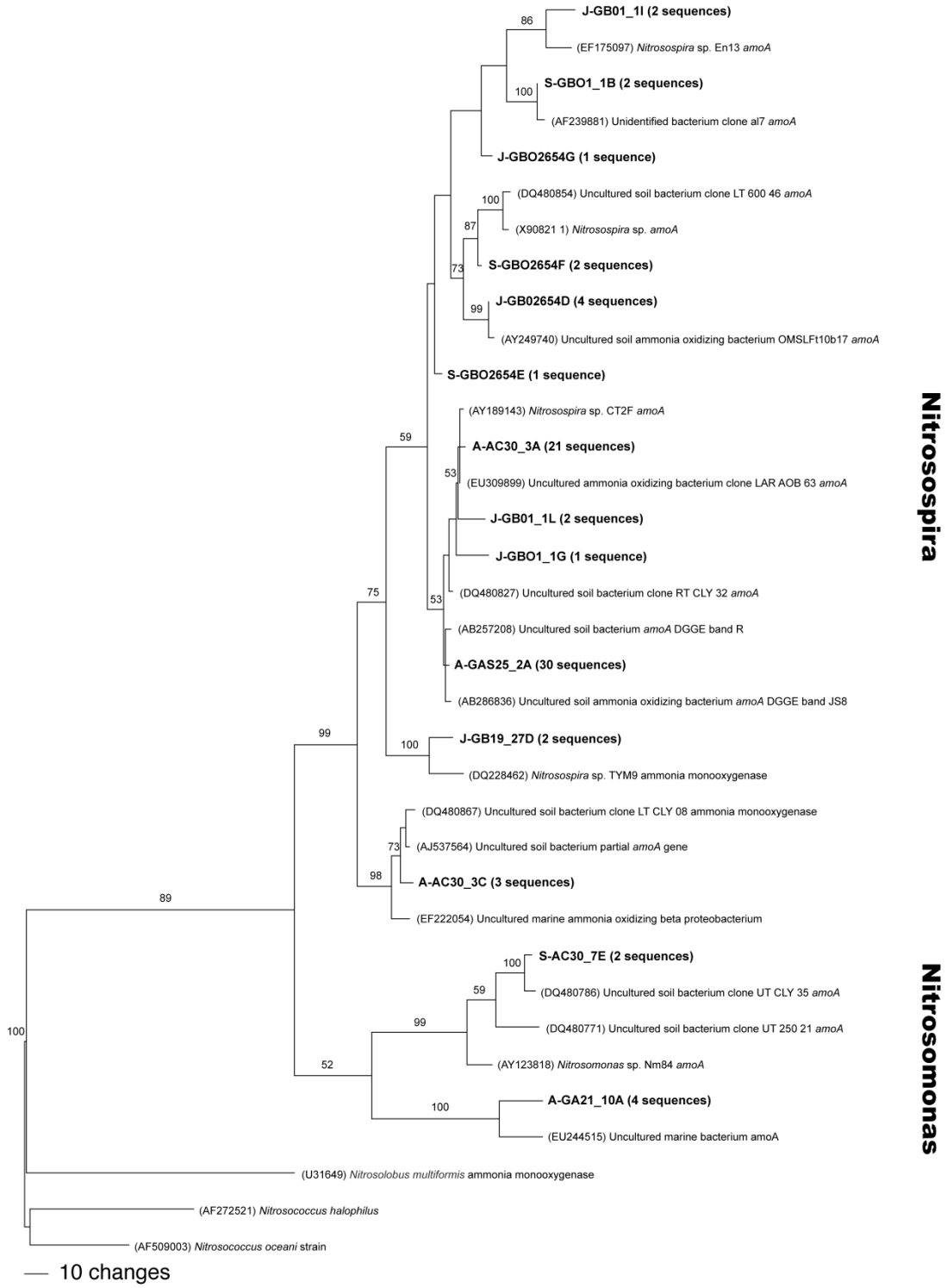
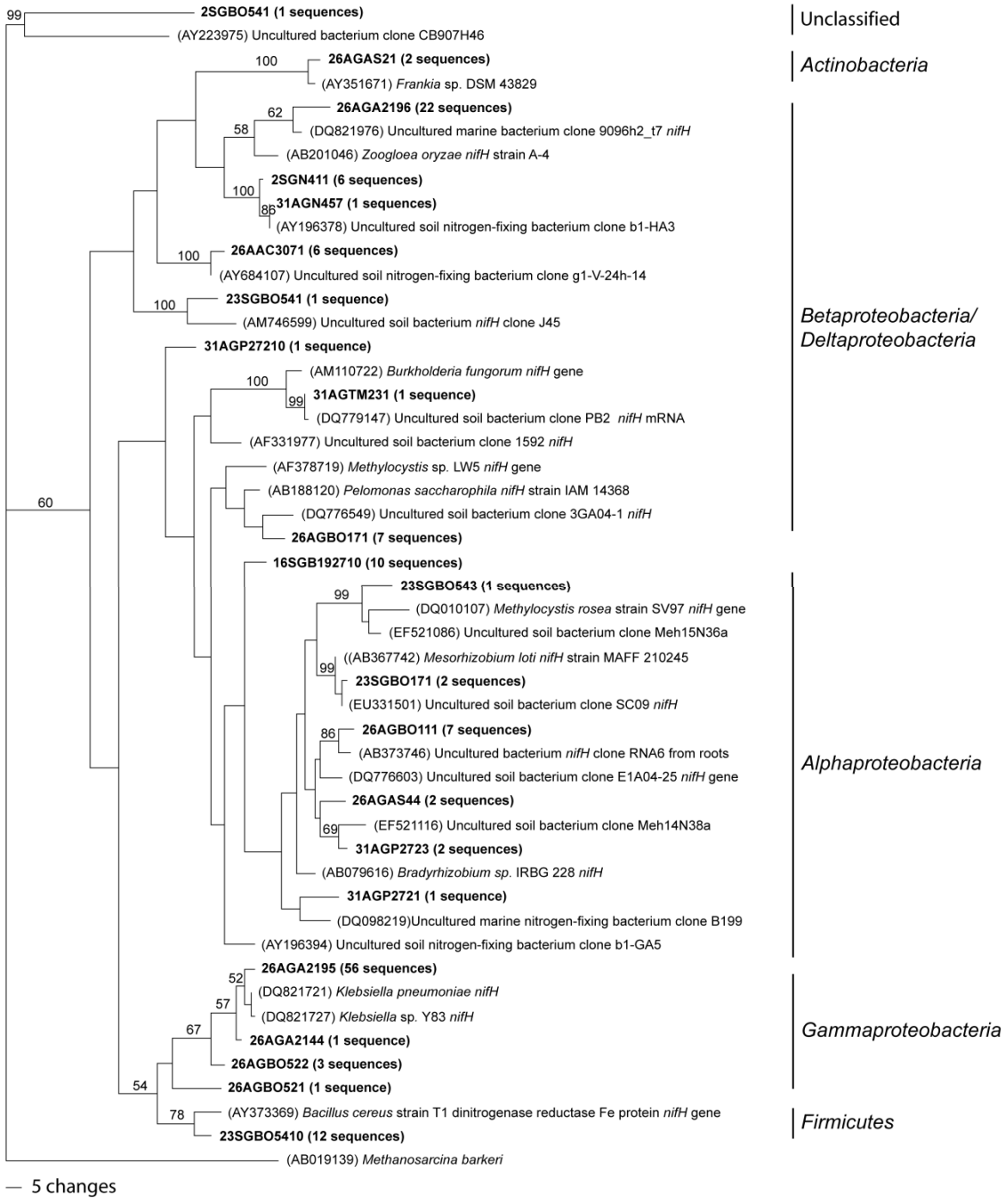


Figure 7. One of the most parsimonious trees of *nifH* OTUs. Bootstrap values for 1000 replicates are shown. Number in parentheses indicates how many sequences fell within the OTU.



Discussion:

The water and soil chemistry showed no statistical differences in the carbon and nitrogen content of material entering the lava tubes based on land use (Table 3). In general, soils from forest and cleared pasture sites within the same biome may be expected to differ in nitrogen and carbon content (Lauber *et al*., 2008; Cenciani *et al*., 2009; Chaer *et al*., 2009). However, no clear trends have emerged from these studies as to what land use regime would have more carbon and or nitrogen in the soil. To our knowledge, there are no previous studies that examine the differences in carbon/nitrogen in water that percolates through pasture soil and forested soil into caves.

The water and soil chemistries were not significantly different for the different land use categories, although it is important to note that flux rates were not measured in this study. This may limit the extent to which the molecular results can be interpreted in terms of the water and soil chemistries. However, there were some trends that indicated differences in the diversity of nitrogen cycling genes in the underlying lava tubes of different use types. Forested lava tubes were more likely to have samples positive for *amoA* than pasture lava tubes; three of the four forested lava tubes were positive, while only two of the six pasture lava tubes contained samples positive for *amoA*. The forested lava tubes also contained a larger number of OTUs, 14 of the 15 OTUs identified (Fig. 5). The same sample from a forested lava tube also had the most bands in the DGGE for both *amoA* and *nifH*, a sample from Gruta Branca Opala. Although these results are not conclusive, there is a trend that the effects of pasture land above the lava tube may be limiting the diversity of AOB. This may be due to a greater flux of ammonia into forested caves due to deeper rooting of the plants above. Further studies would be

needed to conclusively show this difference, and to document the nitrogen flux into these lava tubes.

The lava tubes of Terceira were dominated by *Nitrosospira*-like sequences. *Nitrosospira* are lithoautotrophic bacteria that commonly dominate soil AOB communities (Hayatsu *et al*., 2008). As many of the closest relatives to bacteria living in lava tubes are found in soil environments, it is reasonable to assume that the AOB communities would follow this trend (Northup *et al*., 2008; Garcia *et al*., 2009; Moya *et al*., 2009; Snider *et al*., 2009). *Nitrosospira* has been found to be more diverse in soils that were not treated with urea fertilizer, compared to those that were (Webster *et al*., 2002). The large number of cattle in the pastures may be fertilizing the soil and limiting the diversity of the AOB communities in the pasture land lava tubes.

The dominance of *Nitrosospira*-like sequences in these lava tubes is in contrast to the finding of AOB in Movile Cave, a karst cave in Romania, which was dominated by *Nitrosomonas*-like sequences (Chen *et al*., 2009). However, this study collected water samples that included floating microbial mats, not microbial mats from the walls of the cave. Although *Nitrosomonas* can be found in soils, it is often associated with water samples, especially contaminated water. Movile Cave has a unique atmosphere, with high amounts of hydrogen sulfide, carbon dioxide, methane, decreased oxygen, and is a very different environment than that found in the lava tubes.

One lava tube, Gruta das Agulhas, had only *Nitrosomonas*-like sequences, with no *Nitrosospira*-like sequences. This lava tube is unique in respect to the other lava tubes sampled, as it is located under an urban setting, and opens into the sea. At high tide, waves crash a few meters into the entrance of the lava tube. The sample that was positive

for *amoA* was collected approximately 15 m from the entrance of the lava tube. The proximity to the ocean may influence the microbial communities in this region. Kowalchuk *et al*. (1997) studied the AOB of coastal sand dunes. They found *Nitrosomonas*-like sequences only in dunes closest to the ocean, while *Nitrosospira*-like sequences were found in all dunes. The influence of the ocean spray affected the AOB community in this study, and very well may be influencing the AOB community in Gruta das Agulhas.

The most common sequences recovered from the *nifH* DGGE bands were related to *K. pneumoniae*, which is a Gram negative bacterium belonging to the *Enterobacteriaceae* of *Gammaproteobacteria*. *K. pneumoniae* has a wide range of habitats including soil, vegetation, water, and as pathogens to many mammals. *K. pneumoniae* commonly infects cattle, and was shown to be shed with fecal matter in up to 80% of cows tested in the U.S. (Brisse and van Duijkeren, 2005; Munoz *et al*., 2006). The presence of *K. pneumoniae* in the lava tubes also highlights one of the limitations of this study. We acknowledge that the putative presence of both *amoA* and *nifH* does not confirm activity of these genes within the lava tubes. Furthermore, this study does not allow us to differentiate between different strains of the same species. The source of *K. pneumoniae* within the lava tubes is therefore unknown, and culturing studies should be conducted to better differentiate among strains of this species. Many of the other closest relatives to the *nifH* sequences recovered are found in soil environments, many of which were uncultured. It is common to find closely related sequences from soil environments in 16S rDNA studies of lava tube microbial communities, and *nifH* genes appear to follow this trend as well (Northup *et al*., 2008; Garcia *et al*., 2009; Moya *et al*., 2009;

Snider *et al*., 2009). One close cultured relative that comprised 15% of the recovered sequences was *Zoogloea oryzae*, a nitrogen fixing bacterium isolated from rice paddy soil (Xie and Yokota, 2006). Other species in the *Zoogloea* genus are found in marine environments, as were some of the uncultured closest relatives in this study (Fig. 7). The shallow nature of lava tubes, combined with the probably high hydrologic connectivity from the large amounts of surface rainfall (1000-2300mm) may also be influencing the community structure of *nifH* organisms, allowing a diverse community with close relatives from both soil and marine environments.

The most diverse samples for *nifH* came from Gruta da Branca Opala (GBO) and Gruta dos Buracos (GB). There is nothing in the soil or water chemistry to suggest that these two lava tubes are more similar to each other than any of the other lava tubes (Table 3). Furthermore, Gruta da Branca Opala is under forested land, while Gruta dos Buracos is under pasture land. There may be other environmental factors besides water or soil chemistry that may be affecting microbial community composition. Studies of terrestrial lava flow volcanic rock have shown that the chemical composition of the lava rock influences the resulting bacterial communities (Gomez-Alvarez *et al*., 2007). Future studies should include analysis of underlying basaltic chemistry as a possible determining factor of microbial diversity.

There were twice as many samples positive for *nifH* as there were for *amoA*. This may be due to the low amounts of ammonia coming into the lava tubes in rain-water (Table 3). It is probable that any available nitrogen, from the cattle feces or other sources, is being utilized by the soil plant matter or soil microorganisms, and that little bioavailable nitrogen is entering the lava tubes. The lack of bioavailable nitrogen would

increase the importance of nitrogen fixing bacteria in the establishment and sustainability of microbial mats, as has been suggested for surface lava flows (King, 2007).

Conclusions

This study is, to our knowledge, the first study to detect the *amoA* gene in lava tubes, and the first to detect *nifH* in any cave environment. The *amoA* sequences were mostly *Nitrosospira*-like sequences, which are commonly found in soil, although *Nitrosomonas*-like sequences were also found. Despite the lack of significant differences in the water and soil chemistries in regards to land use, there were unique OTUs for both *amoA* and *nifH* in the pasture and forested caves. *nifH* was more widely found, and was dominated by a *K. pneumoniae*-like sequences, which is most likely coming from bovine fecal matter washing into the lava tubes. Although this study does not show that these genes are being expressed, it provides a framework upon which futures studies can build.

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Chapter 4

Conclusions

Lava tubes contain a diverse community of bacteria living on the walls and ceilings. Visually, the bacterial mats in Hawai‘i and the Azores are very similar, but genetically, they were very different from each other. The lava tubes of Hawai‘i had greater diversity than those in the Azores, which is consistent with similar studies on invertebrates and plants in the two locations (Wittaker et al., 2008; Borges et al., 2009; Price, 2009). This study also found novel bacteria belonging to the phylum *Actinobacteria* in both Hawai‘i and the Azores that were between 85% and 97% similar to any known sequence in the GenBank database. There were more *Actinobacteria* sequences from yellow samples than from white. Furthermore, there was strong evidence for a geographic influence on diversity. Although many of the same phyla were found at both locations, when compared at the species level there was clear separation of lava tube bacterial communities as a whole from Hawai‘i and those from the Azores. None of the other abiotic factors tested for, such as elevation, temperature, precipitation, color of microbial mat and land use, showed clear patterns. Color is a visual distinguishing factor, and mats of the same color can look identical to the unaided eye. The color of the mat may be due to other microbes such as fungi, which were not included in this study, or to compounds produced by microorganisms present. The diversity differences between Hawai‘i and the Azores, may be due to unique chemical composition of the underlying lava that is influencing the resulting microbial communities, which will be investigated in future studies.

The 16S rDNA diversity found in the Azores will aid in concurrent culturing efforts to find new types of antibiotics and to describe new species. By determining what phyla of bacteria are present in the lava tubes, better media can be designed to culture a greater variety of the bacteria, increasing the chances of finding novel antibiotics and new species, both of which may provide strong rationales for preserving these valuable ecosystems. *Actinobacteria* are known antibiotic producers, and the results from this study suggest that lava tubes are an ideal setting in which to find novel types of these bacteria.

In the Azores, the microorganisms present in the lava tubes also contain the nitrogen fixation gene *nifH,* and the ammonia oxidation gene *amoA*. This study is the first to show the presence of these two genes in lava tubes, extending the habitats where these two genes are found. It is also the first to examine the presence of *nifH* containing microorganisms in caves of any kind. The scope of this study did not allow the determination of whether these two genes were being expressed in the lava tubes, which would be the next step in determining the ecological role of nitrogen cycling bacteria in lava tubes. However, this study did highlight some important environmental factors regarding the diversity and distribution of both *nifH* and *amoA*. The *nifH* samples were dominated by *Klebsiella pneumonia*-like sequences, which could be from bovine fecal matter entering the lava tubes through percolating rainwater. This suggests that there is a high amount of connectivity between the surface and the lava tubes. The results from the *amoA* survey showed there was more diversity of ammonia oxidizers in lava tubes found under forested lands rather than pasture lands, again suggesting that surface conditions play a role in determining the underlying bacterial community.

These two studies together provide a scientific basis for conservation efforts to preserve the lava tube ecosystems. One of the lava tubes studied in Hawai'i is threatened by the construction of a school above it, while several of the lava tubes in the Azores lie beneath extensive pastures grazed by cattle. The high amount of diversity and the discovery of novel bacteria in both the Azores and Hawai‘i suggest that lava tubes are valuable ecosystems that harbor new species of microorganisms that may play key roles in the ecosystem. The finding of extremely novel *Actinobacteria* helps in focusing the search for new types of antibiotics and the expansion of the Tree of Life with new species. The nitrogen cycling studies suggest that surface uses can greatly influence the types of bacteria capable of carrying out ammonia oxidation able to live within the lava tubes. The presences of *K. pneumonia*-like sequences across the Azorean island suggest that the presence of cattle above lava tubes has far reaching influences through the movement of water percolating from manure-laden pastures into the lava tubes. Currently, in both Hawai‘i and the Azores, lava tubes are often used as garbage dumps, and the impact of this human disturbance is not yet known on the bacterial communities. Efforts to educate the public on the unique nature of the lava tubes are underway in the Azores, and these studies provide scientific evidence with which to influence local human utilization of the lava tubes.

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