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

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Lava caves are an understudied ecosystem in the subterranean world, particularly in regard to nitrogen cycling. The diversity of ammonia oxidation (*amoA*) and nitrogen fixation (*nifH*) genes in bacterial mats collected from lava cave walls on the island of Terceira (Azores, Portugal) was investigated using denaturing gradient gel electrophoresis (DGGE). A total of 55 samples were collected from 11 lava caves that were selected with regard to surface land use. Land use types above the lava caves were categorized into pasture, forested, and sea/urban, and used to determine if land use influenced the ammonia oxidizing and nitrogen fixing bacterial communities within the lava caves. The soil and water samples from each lava cave were analyzed for total organic carbon, inorganic carbon, total nitrogen, ammonium, nitrate, phosphate and sulfate, to determine if land use influences either the nutrient content entering the lava cave or the nitrogen cycling bacteria present within the cave. *Nitrosospira*-like sequences dominated the ammonia-oxidizing bacteria (AOB) community, and the majority of the diversity was found in lava caves under forested land. The nitrogen fixation community was dominated by *Klebsiella pneumoniae*-like sequences, and diversity was evenly distributed between pasture and forested land, but very little overlap in diversity was observed. The results suggest that land use is impacting both the AOB and the nitrogen fixing bacterial communities.

**Keywords:** *amoA*, bacteria, lava caves, *nifH*, nitrogen cycling

## Introduction

The cycling of nitrogen provides energy and nutrients 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 (Collins et al. 2008; McCarthy et al. 2007; Prosser and Nicol 2008; Ross et al. 2009; Sooksa-nguan et al. 2009; Treusch et al. 2005; Venter et al. 2004). 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 semiclosed environments, nitrogen fixation by *Bacteria* and *Archaea* can be the main source of bioavailable nitrogen for other organisms, while ammonia from organic matter mineralization can be a source of energy for chemolithotrophic organisms. Studies of lithotrophic ammonia oxidation in subsurface environments are rare (Chen et al. 2009; Simon and

Benfield 2002; Spear et al. 2007). More generally, there has been little study of nitrogen cycling or the diversity of the organisms that carry out nitrogen transformations in subsurface environments.

We investigated the diversity of *amoA* and *nifH* genes that encode the enzymes that mediate nitrogen transformation processes in microbial mats collected in lava caves of Terceira, Azores, Portugal. Nitrogenase, the enzyme responsible for the fixation of nitrogen, is partially encoded by the gene *nifH* (Dean and Jacobson 1992; Ueda et al. 1995). Nitrogen fixation is an energetically expensive process that is performed 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; Northup et al. 2003).

The limiting step in nitrification, the transformation of ammonia to nitrate, is the conversion of  $\text{NH}_4^+$  to  $\text{NH}_2\text{OH}$  (Kowalchuk and Stephen 2001). 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

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ferromanganese deposits in Spider and Lechuguilla Caves in New Mexico, giving evidence of the potential 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 biologically unavailable. However, the presence of ammonia may be an indication of nitrogen fixation.

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 (Barton and Jurado 2007). 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 nonaquatic cave environments (Fliermans and Schmidt 1977; Jones et al. 2008; Northup et al. 2003, Snider 2010). 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 caves are a unique subset of subsurface environments, and are formed during a volcanic eruption. One major mechanism for the formation of lava caves is that as the erupting lava cools on the surface, a river of molten lava continues to flow underneath. When the eruption ceases, the river of lava flows downhill, leaving an empty tube behind. Lava caves are generally more shallow subterranean environments than limestone caves (Palmer 2007). Their shallowness, combined with the tendency for lava caves 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 caves have long been described, but the composition and diversity of the mats is just being explored with culture-independent techniques (Garcia et al. 2009; Moya et al. 2009; Northup et al. 2008, 2011; Snider 2010; Snider et al. 2009; Staley and Crawford 1975; Stoner and Howarth 1981). A comparison of 16S rRNA gene bacterial diversity from lava caves in Hawai'i and New Mexico in the United States, and the Azores, Portugal, showed Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Acidobacteria present in all caves studied (Northup et al. 2011).

In an analysis of 16S rRNA bacterial gene clone libraries, Actinobacteria such as *Frankia* and *Alphaproteobacteria* such as *Mesorhizobium* sequences are found (Hathaway unpublished data). Additionally *Nitrosospora* sp. and *Nitrosomonas* sp. were found, indicating that there is the possibility to do both nitrogen fixation and ammonia oxidation within lava caves (Hathaway unpublished data). 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 the presence of key genes involved in nitrogen fixation and ammonia oxidation in 11 lava caves on Ter-

ceira Island in the Azores, Portugal. Terceira has lava caves across the island, some underneath extensive cow pastures and some underneath forested areas of both native and exotic plants. The soils overlying the lava caves are classified as andisols (Madeira et al. 2007). Because of the high precipitation rate and high hydrologic connectivity between the lava caves and the surface, we hypothesized that nutrient inputs to lava caves 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 caves 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 caves were selected to represent a range of elevation, annual precipitation, and surface land use (Figures 1 and 2; Table 1).

### Sample Collection

Samples of microbial mats were collected using aseptic methods in February and June 2008. Samples of yellow, white and tan, the most frequently observed colors of microbial mats, were collected from each cave, as well as microbial mats of less frequently observed mat colors, such as pink, grey and black. Samples were selected for collection based on uniformity of color (see Figure 2 for an example microbial mat). 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. One water sample was collected per cave with the exception of Gruta de Balcoes, where two water samples were collected. Results from the analysis of the water from Gruta de Balcoes were averaged. The limited water sampling was due to the scarcity of dripping water in the caves. Samples were kept at 4°C until analysis. Temperature and humidity data (wet bulb/dry bulb) were collected throughout the cave with an IMC Digital Thermometer probe (Wittenberg, WI, USA). This instrument does not allow for accurate humidity measurements above 95%, but can be calibrated, which increased accuracy below 95%.

### Water and Soil Chemistry

Dissolved organic carbon (DOC) in infiltrating water was collected and passed through a 0.45  $\mu$ m 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,

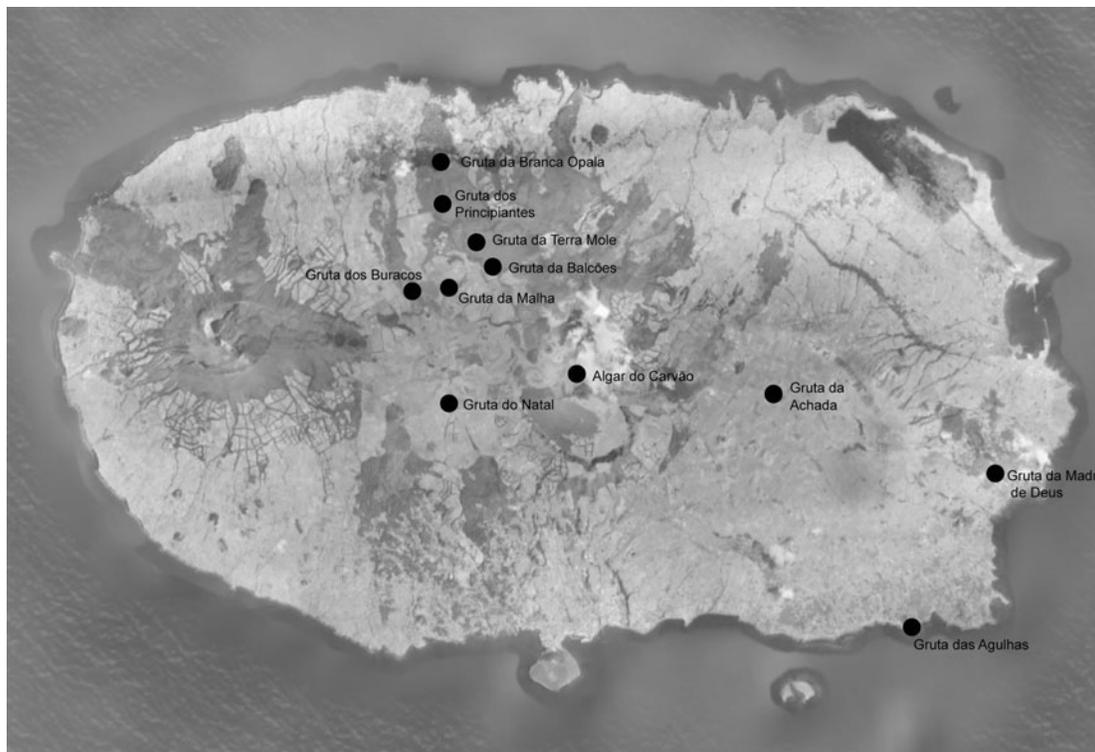


Fig. 1. Map of Terceira Island, with the location of the lava caves.



Fig. 2. Images showing different land use practices above the cave and bacterial mats within the lava caves 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. © Kenneth Ingham. Reproduced by permission of Kenneth Ingham. Permission to reuse must be obtained from the rightsholder.

**Table 1.** Abiotic factors associated with each cave

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	Pasture

Land use describes how the land above the lava cave is used by humans or the nature of the vegetation above the lava cave where no human use is present.

nitrate, and phosphate were analyzed using a Dionex Ion Chromatograph DX-100 (Dionex, Sunnyvale, CA, USA) as described in Pfaff et al. (1997). The amount of ammonia in the water samples was analyzed using a Technicon (1973) AutoAnalyzer II (Technicon, Tarrytown, NY, USA).

One soil sample was collected from the floor near the entrance of each lava cave and one soil sample was collected from deeper within the interior of the lava cave. 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 cave were tested for statistically significant differences then averaged.

#### DNA Extraction and PCR Amplification

DNA was extracted and purified from 55 samples from the 11 lava caves 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- $\mu$ L reaction mixture containing 1 $\times$  PCR buffer with 2 mM Mg<sup>2+</sup>, 0.2 mM each dNTP, 0.4  $\mu$ M of each primer, 5  $\mu$ g BSA and 0.75 U of TaKaRa Ex Taq (TaKaRa, Shiga, Japan).

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 50- $\mu$ L reaction mixture containing 1 $\times$  PCR buffer with 1.5 mM Mg<sup>2+</sup>, 0.8  $\mu$ M of each primer, 0.2 mM each dNTP, 5  $\mu$ g 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  $\mu$ M.

#### Denaturing Gradient Gel Electrophoresis

For any sample that was positive for the gene of interest, 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 using the same PCR conditions as previously described (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 1 $\times$  TAE buffer on a CBS DGGE-1001 (CBS Scientific, Del Mar, CA). Gels were run in duplicate, with one for band excising and one for imaging.

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, reamplified 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 reamplified were then sequenced with Big Dye Terminator v1.1 using 5  $\mu$ l 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 operational taxonomic unit (OTU) using mothur version 1.17 (Schloss et al. 2009). Community analyses were also performed 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. Sequences were submitted to the GenBank database and assigned accession numbers HM461331-HM461407 for *amoA* sequences, and HM461408-HM461553 for *nifH* sequences.

**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, 1 min 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 GCCCCGCCCCGCCCC 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 AQER PolF GC	TGCGAYCCSARGCBGACTC GACGATGTAGATYTCCTG CGCCCGCCGCGCGGGCGGGC GGGGCGGGGGCACG GGGGGTGCGAYCCSAR GCBGACTC	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) Poly et al. (2001) Demba-Diallo et al. (2004)

## 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  $p = 0.99$ , soil  $p = 0.59$ ) and nitrogen (water  $p = 0.49$ , soil  $p = 0.93$ ) in material entering the lava cave. None of the samples were statistically significantly different in terms of the amount of  $\text{SO}_4^{2-}$  ( $p = 0.80$ ),  $\text{NO}_3^-$  ( $p = 0.72$ ) and  $\text{NH}_4^+$  ( $p = 0.91$ ) as determined by the water analysis. The only element that was statistically significantly different was  $\text{PO}_4^-$  ( $p = 0.017$ ).

### Diversity of amoA Genes

A total of 14 (25%) of the 55 samples tested were positive for the *amoA* gene, from six of the 11 lava caves, 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 (Figure 3, Table 4). Using a 97% similarity cutoff, 15 unique OTUs were designated. Ten OTUs were found exclusively in lava caves underlying forested land, one exclusively in a lava cave under a pasture, three were shared between lava caves under pastures and lava caves under forests, and one was shared between lava caves under forests and the sea/urban lava cave (Figure 4A). Thirteen of the 15 unique OTUs were similar to known species of *Nitrosospira*, and the other two OTUs were similar to *Nitrosomonas* sequences (Figure 5). 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 cave that opens into the sea. There were no OTUs shared by all samples.

### Diversity of nifH Genes

A total of 30 of the 55 (55%) samples screened were positive for the *nifH* gene, from all 11 lava caves 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 (Figure 6, Table 4). Nine of the OTUs were unique to the lava caves under forested land, seven were unique to the lava cave under pasture land, and one OTU was unique to the sea/urban lava cave (Figure 4B).

There were two OTUs shared by the lava caves under forested and pasture lands, one shared by the pasture and sea/urban lava cave, one shared by the forested and sea/urban lava cave, and one OTU shared by all three land use categories, a *Klebsiella pneumoniae*-like sequence. Sixty-one of the 146 bands (41.7%) of the sequences recovered were identified as *K. pneumoniae*-like sequences. *K. pneumoniae*-like sequences were found in 13 samples from eight different lava caves. 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, *Alphaproteobacteria* (25 sequences), *Betaproteobacteria* (38 sequences), *Deltaproteobacteria* (2 sequences) and *Gammaproteobacteria* (67 sequences) (Figure 7). There were no OTUs shared by all samples.

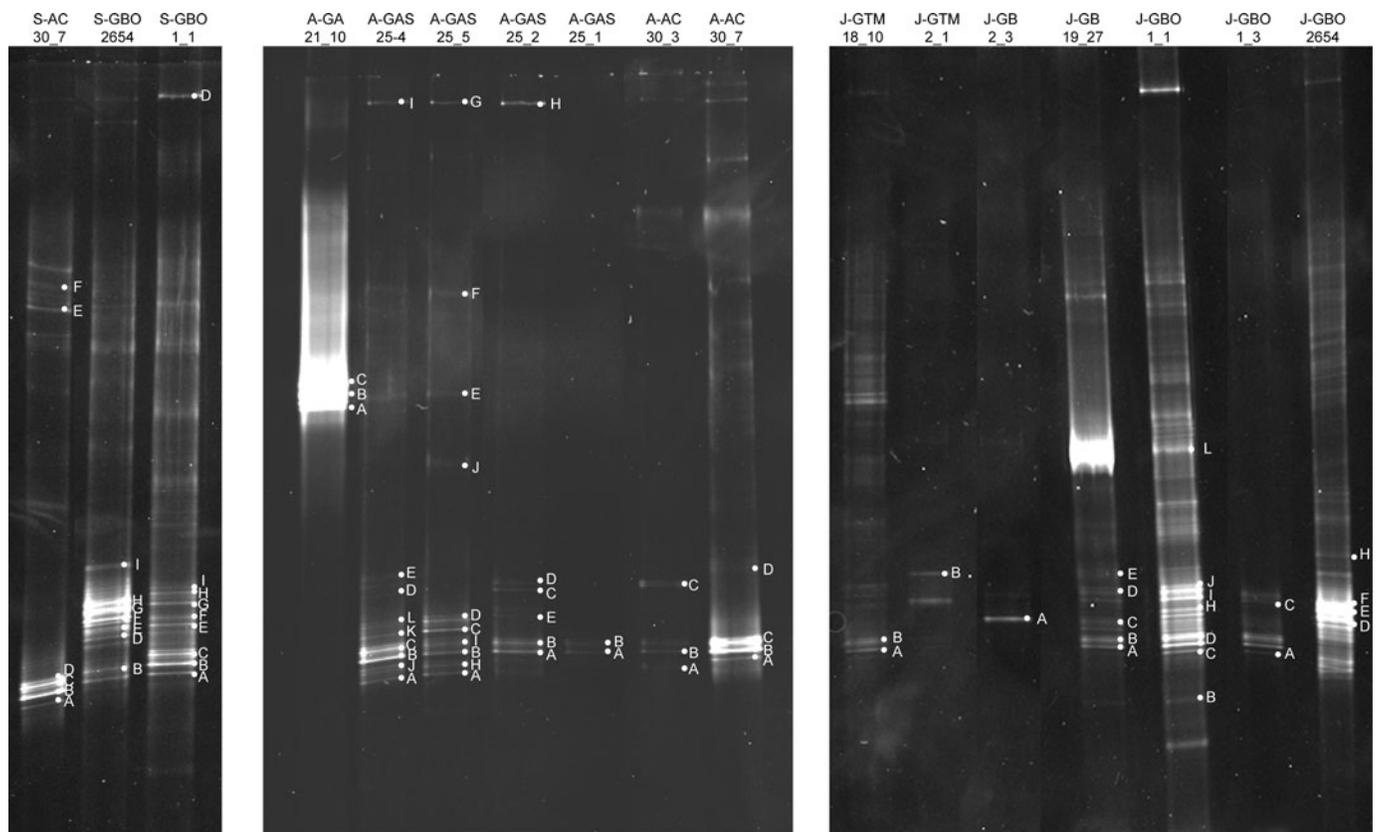
**Table 3.** Water and soil chemistry

Cave	Abbreviation	Water							Soil		Land Use
		PO <sub>4</sub> <sup>-</sup>	SO <sub>4</sub> <sup>2-</sup>	TOC	IC	NO <sub>3</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	TN	% N	%C	
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

**Discussion**

The water and soil chemistry showed little statistical differences in the nutrient content of material entering the lava caves based on land use (Table 3). This is probably due to small sample sizes, however the high p values for most nutrients indicate that that samples probably are not different. The only exception is the phosphorus levels, with a p = 0.017. In

general, soils from forest and cleared pasture sites within the same biome may be expected to differ in nitrogen and carbon content (Cenciani et al. 2009; Chaer et al. 2009; Lauber et al. 2008). However, no clear trends have emerged from these studies as to the magnitude of soil carbon and nitrogen responses to land use regime. 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



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

**Table 4A.** *amoA* DGGE band identification

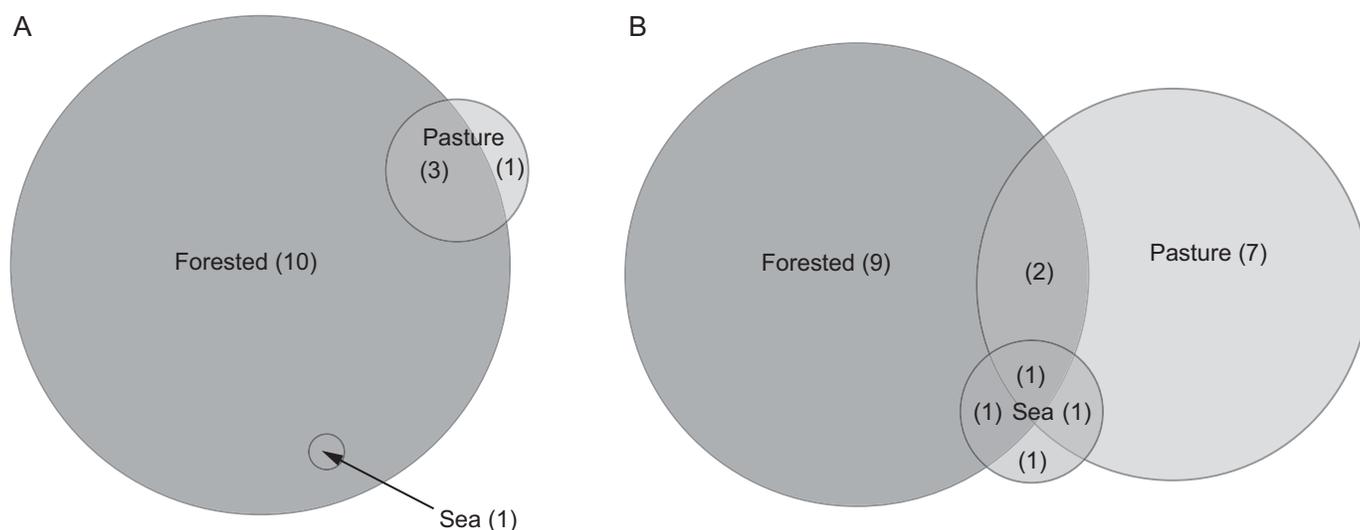
Sample Name	Number of Bands Sequenced	Number of Phylotypes Obtained	Phylogenetic Groups Identified	Phylotype Number(s)	Phylotype Accession Number(s)
AC30-3	3	2	<i>Nitrosospira</i> , <i>Nitrosospira</i>	1,2	HM461331, HM461333
AC30-7	6	2	<i>Nitrosospira</i> , <i>Nitrosomonas</i>	1, 11	HM461331, HM461391
GA21-10	3	1	<i>Nitrosomonas</i>	3	HM461337
GAS25-1	2	1	<i>Nitrosospira</i>	1	HM461331
GAS25-2	5	1	<i>Nitrosospira</i>	4	HM461342
GAS25-4	7	1	<i>Nitrosospira</i>	4	HM461342
GAS25-5	7	2	<i>Nitrosospira</i> , <i>Nitrosomonas</i>	3,4	HM461337, HM461342
GB02-3	1	1	<i>Nitrosospira</i>	2	HM461333
GB19-27	5	3	<i>Nitrosospira</i> , <i>Nitrosospira</i> , <i>Nitrosospira</i>	1,4,8	HM461331, HM461342, HM461366
GBO01-1	16	6	<i>Nitrosospira</i> , <i>Nitrosospira</i> , <i>Nitrosospira</i> , <i>Nitrosomonas</i>	1,4,5,6, 9,12	HM461331, HM461342, HM461375, HM461394
GBO01-3	1	1	<i>Nitrosospira</i>	4	HM461342
GBO26-54	16	5	<i>Nitrosospira</i> , <i>Nitrosospira</i> , <i>Nitrosospira</i>	4,7,10, 13, 14	HM461342, HM461378, HM461381
GTM18-10	2	1	<i>Nitrosospira</i>	1	HM461331
GTM2-1	1	1	<i>Nitrosospira</i>	2	HM461333

caves. Future studies will gather additional data points with which to explore these parameters under different seasonal time frames.

It is important to note that although the water and soil chemistries were not significantly different for the different land use categories, 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 interesting trends that indicated differences in the diversity of nitrogen cycling genes in the underlying lava caves of different land use types. Lava caves under forested land were more likely to have samples positive for the presence of *amoA* genes than lava caves under pastures; three of the four

lava caves under forested land were positive, yet only two of the six lava caves occurring under pastures contained samples positive for *amoA*.

The lava caves under forested land also contained a larger number of OTUs, 14 of the 15 OTUs identified (Figure 4). The same sample from a forested lava cave also had the most bands in the DGGE gel for both *amoA* and *nifH*, a sample from Gruta Branca Opala. Although these results are not conclusive, they suggest that lava caves that lie below forested land, in contrast to those below pastures, contain a greater diversity of AOB. We will investigate whether this AOB diversity under forested lands is also observed in our other field sites in Hawai'i and New Mexico in future studies.



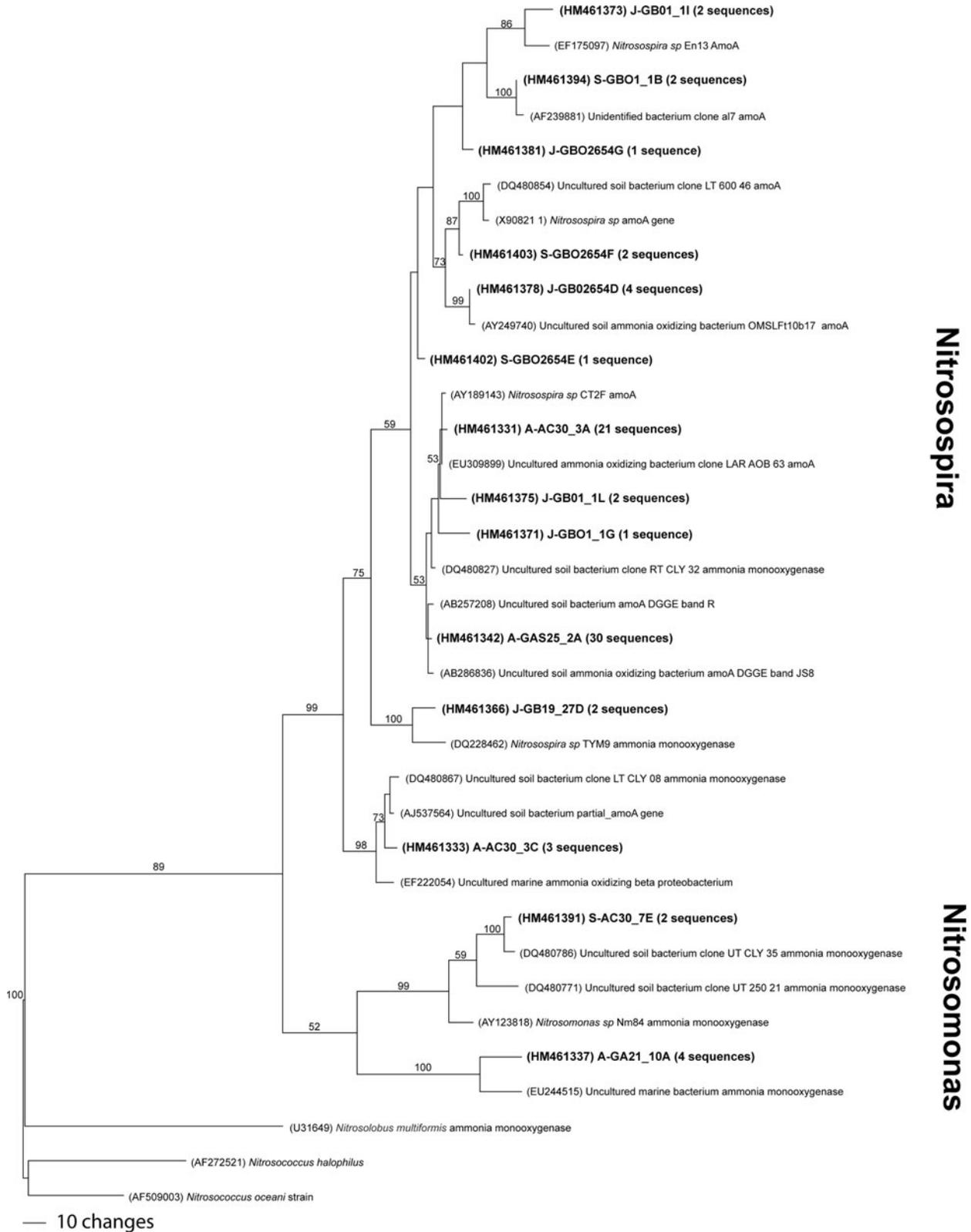
**Fig. 4.** Venn diagrams showing unique and overlapping OTUs for (A) *amoA* and (B) *nifH* DGGE results, based on the land use above the lava cave. The number in the parentheses indicated the number of unique OTUs for that land use category.

**Table 4B.** *nifH* DGGE band identification

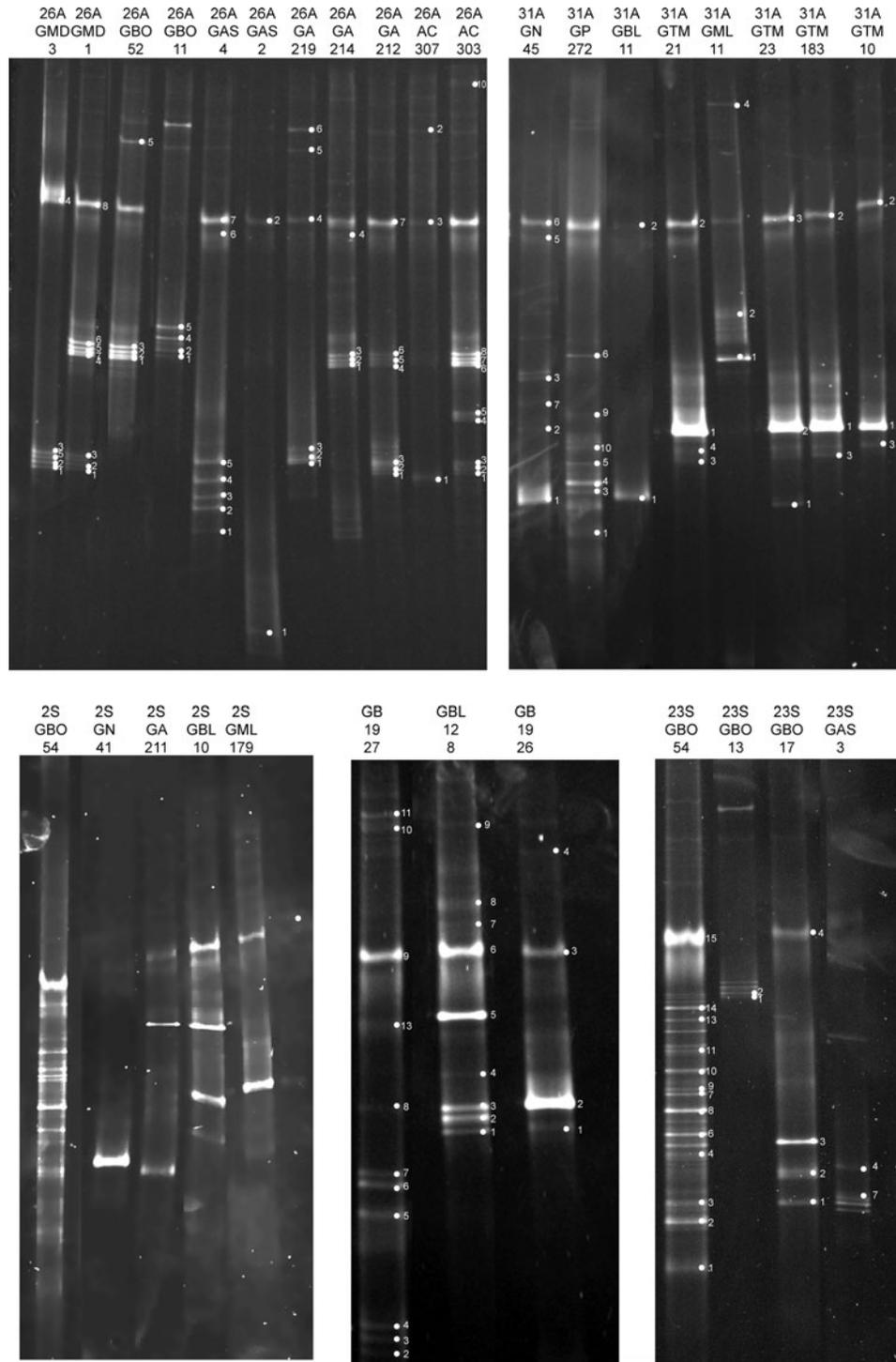
Sample Name	Number of Bands Sequenced	Number of Phylotypes Obtained	Phylogenetic Groups Identified	Phylotype Number(s)	Phylotype Accession Number(s)
AC30—3	9	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
AC30-7	3	1	<i>Gammaproteobacteria</i>	12	HM461433
GA21-1	3	1	<i>Gammaproteobacteria</i>	12	HM461433
GA21-2	7	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
GA21-4	4	2	<i>Gammaproteobacteria (Klebsiella)</i> , <i>Gammaproteobacteria (Klebsiella)</i>	7,8	HM461451, HM461446
GA21-9	6	2	<i>Gammaproteobacteria (Klebsiella)</i> , <i>Gammaproteobacteria</i>	7,22	HM461451, HM461452
GAS25-2	2	1	<i>Actinobacteria (Frankia)</i>	2	HM461453
GAS25-3	2	1	<i>Betaproteobacteria (Burkholderiales)</i>	15	HM461466
GAS25-4	7	2	<i>Alphaproteobacteria, Alphaproteobacteria</i>	20,21	HM461462, HM461458
GB19-26	4	2	<i>Gammaproteobacteria (Klebsiella)</i> , <i>Gammaproteobacteria</i>	7,22	HM461451, HM461452
GB19-27	10	1	<i>Betaproteobacteria</i>	16	HM461535
GBL12-10	4	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
GBL12-11	2	1	<i>Betaproteobacteria (Burkholderiales)</i>	15	HM461466
GBL12-8	9	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
GBO01-1	4	2	<i>Gammaproteobacteria (Klebsiella)</i> , <i>Alphaproteobacteria</i>	7,20	HM461451, HM461462
GBO01-3	2	1	<i>Gammaproteobacteria (Klebsiella)</i>	5	HM461415
GBO01-7	7	4	<i>Gammaproteobacteria (Klebsiella)</i> , <i>Gammaproteobacteria (Klebsiella)</i> , <i>Deltaproteobacteria, Alphaproteobacteria</i>	7,9,15,18	HM461451, HM461470, HM461466, HM461410
GBO26-52	4	3	<i>Gammaproteobacteria (Klebsiella)</i> , <i>Gammaproteobacteria (Klebsiella)</i> , <i>Gammaproteobacteria (Klebsiella)</i>	6,7,9	HM461469, HM461451, HM461470
GBO26-54	10	4	Unclassified, <i>Deltaproteobacteria</i> , <i>Firmicutes (Bacillus)</i> , <i>Betaproteobacteria</i>	1 3,5,13	HM461493, HM461414, HM461415, HM461420
GMD20-1	8	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
GMD20-3	5	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
GML17-11	3	1	<i>Gammaproteobacteria</i>	22	HM461452
GML17-9	2	1	<i>Gammaproteobacteria (Klebsiella)</i>	7	HM461451
GN04-1	1	1	<i>Gammaproteobacteria</i>	10	HM461500
GN4-5	5	2	<i>Gammaproteobacteria</i> , <i>Gammaproteobacteria</i>	10,11	HM461500, HM461511
GP27-2	6	5	<i>Betaproteobacteria, Gammaproteobacteria (Klebsiella)</i> , <i>Alphaproteobacteria, Alphaproteobacteria</i>	4,7,17,19,21	HM461513, HM461451, HM461512, HM461516, HM461458
GTM2-1	4	1	<i>Gammaproteobacteria</i>	22	HM461452
GTM2-3	3	2	<i>Betaproteobacteria (Burkholderiales)</i> , <i>Gammaproteobacteria</i>	14, 22	HM461528, HM461452
GTM018-10	3	1	<i>Gammaproteobacteria</i>	22	HM461452
GTM18-3	3	1	<i>Gammaproteobacteria</i>	22	HM461452

Factors such as the nature of the soil microbial community above the lava cave and the effect of fertilizers on these communities may influence the AOB communities in the lava caves. A future study will compare and contrast the soil microbial

communities above the caves with those within the caves. Other factors such as the pH of the soil, vegetation, soil nutrient levels and salinity, have been shown to influence the diversity of *Nitrosopira* in different soil communities (Fierer et al. 2009).



**Fig. 5.** 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.



**Fig. 6.** DGGE image of *nifH* gels. Sample names are along the top and individual bands sequenced are labeled within each gel. 26A, 31A, 2S, 16S and 23S designate different gel runs.

The difference in diversity may also be due to a greater flux of ammonia into forested caves due to deeper rooting of the plants above. Further studies are needed to conclusively show this difference, and to document the nitrogen flux into these lava caves.

The lava caves of Terceira were dominated by *Nitrosospira*-like sequences. This result may be influenced by primer bias, but the sea cave did show non-*Nitrosospira*-like sequences, rather than *Nitrosospira*-like sequences. *Nitrosospira* are lithoautotrophic bacteria that commonly dominate

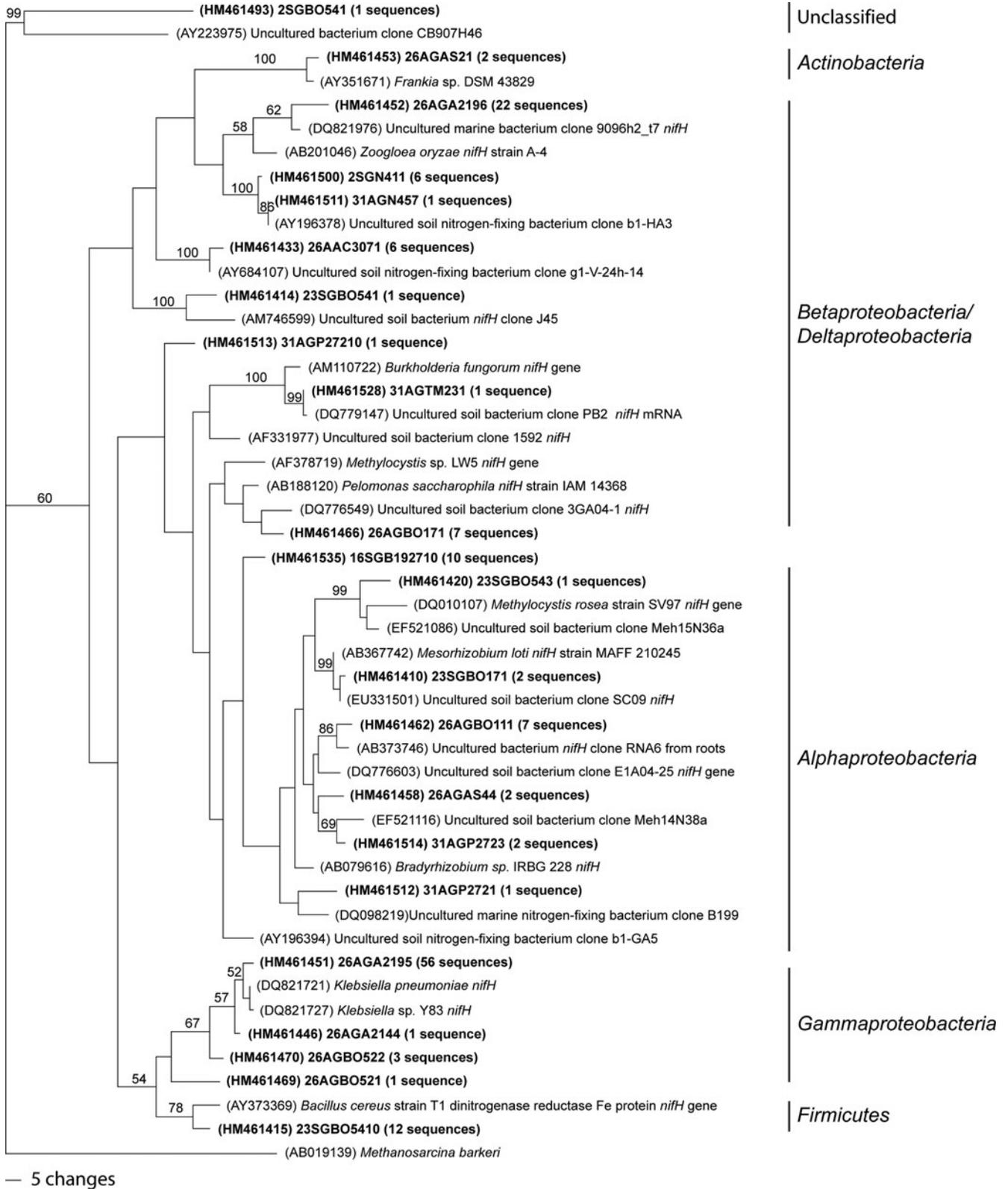


Fig. 7. One of the most parsimonious trees of *nifH* OTUs. Bootstrap values for 1000 replicates are shown. Number in parentheses indicates how many sequences fell within the OTU.

terrestrial AOB communities (Hayatsu et al. 2008). As many of the closest relatives to bacteria living in lava cave microbial mats also are found in soil environments, an obvious colonization source for the lava caves, it is reasonable to assume that the AOB communities would follow this trend (Garcia et al. 2009; Moya et al. 2009; Northup et al. 2008; Snider 2010; Snider et al. 2009). However, we have also found that lava caves contain many novel organisms (some less than 85% similarity to known sequences), a finding that we are exploring to determine differences among overlying soil communities with those found in the underlying caves (Hathaway unpublished data). *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 use of chemical fertilization and the large number of cattle in the pastures may be limiting the diversity of the AOB communities in the pasture land lava caves.

Four of the 15 OTUs, representing 54 of the 78 sequences, group within *amoA* Cluster 1, which is commonly found in marine environments. However, studies have also found representatives from this cluster in soil environments (Fierer et al. 2009; Schmidt et al. 2007). This may indicate the strong marine influence on the terrestrial island soils in the lava caves, or may be due to the moist nature of lava caves, most of which have 95–100% humidity year-round. The other OTUs grouped with *Nitrosospira* Clusters 2, 3 and 4, all of which are commonly found in soil environments (Hayatsu et al. 2008) and volcanic soils (Cluster 3) in Hawai'i (King and Nanba 2008).

Although multiple studies have found that several environmental factors can influence the AOB composition, similar ecosystem types do not appear to harbor the same AOB communities (Fierer et al. 2009). Furthermore, Fierer et al. (2009) found no significant correlations between amount of nitrogen available and AOB composition across multiple soil ecosystems. This may indicate that although lava caves are similar on the ecosystem scale, each is unique and will harbor its own specific community of AOB.

The dominance of *Nitrosospira*-like sequences in these lava caves is in contrast to the finding of AOB in Movile Cave, a closed-system carbonate cave in Romania near the Black Sea, 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, has been shown to be isolated from surface influences (Sarbu et al. 1996), and is a very different environment than that found in the lava caves.

One lava cave, Gruta das Agulhas, had only *Nitrosomonas*-like sequences, with no *Nitrosospira*-like sequences. This lava cave is unique in respect to the other lava caves 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 cave. The sample that was positive for *amoA* was collected approximately 15 m from the entrance of the lava cave. 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, and it is thought that salinity affects the type of AOB present in an ecosystem. The influence of the ocean spray affected the AOB community in the Kowalchuk et al. study (1997), 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. In nonclinical environments, *K. pneumoniae* is known to contribute to both biochemical and geochemical processes and can be a major component of the microbiota (Brisse et al. 2006). Hunter et al. (2004) showed that coliforms such as *Escherichia coli* can persist in cave environments, despite the low nutrients available. In a more clinical setting, *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). Due to the large number of cattle that inhabit Terceira, it is not hard to imagine them as a source of the *K. pneumoniae* in the caves.

In contrast to our *nifH* finding, no *Klebsiella*-like sequences were recovered in our recent 16S rRNA analyses of the same lava caves (Hathaway, unpublished data). This may be due to the limited number of sequences, approximately 60, from each clone library. Moreover, in a more extensive survey of the caves from Lava Beds National Monument, California, USA using 454 sequencing no *Klebsiella*-like sequences were recovered in microbial mats or surface soils above (Northup unpublished results). However, there are no cattle grazing above these caves. We believe that future 454 sequencing of the Terceira microbial mats will help to answer the question of whether *K. pneumoniae* is really present in the caves. The presence of *K. pneumoniae* in the lava caves 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 caves. The *K. pneumoniae*-like sequences could be remnant DNA from surface contaminants, and not actual living cells in the caves. Future studies will include next generation sequencing to increase the number of sequences analyzed from each cave. This will allow for more in depth analysis of the species in the cave. Future work should also include RNA studies to determine which, if any, of the genes found in this study are actively being expressed in the caves.

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 rRNA studies of lava cave microbial communities, and *nifH* genes appear to follow this trend as well (Garcia et al. 2009; Moya et al. 2009; Northup et al. 2008, 2011; Snider et al. 2009; Snider 2010). 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 (Figure 7). This genus also has been found in our recent

analyses of microbial mats from Lava Beds National Monument, California, USA (Northup, unpublished results). The shallow nature of lava caves, combined with the probably high hydrologic connectivity and the large amounts of surface rainfall (1000–2300 mm) 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 caves are more similar to each other than any of the other lava caves (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 will include analysis of underlying basaltic substrate 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 caves 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 caves. 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).

This study is, to our knowledge, the first report to describe the *amoA* gene diversity in lava caves, and the first to detect the presence of the *nifH* gene 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 caves. Although this study does not show that these genes are being expressed, it provides a framework upon which future studies can build and is the first step in piecing together the microbial role in cycling nitrogen within caves. Future studies will explore other nitrogen cycle genes within the caves and compare these findings to the diversity found in soils that overlie the caves, and will investigate whether these processes are actually occurring within the caves.

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