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ORIGINAL PAPER

Bacterial Diversity and Composition in Oylat Cave (Turkey) with Combined Sanger/Pyrosequencing Approach

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Abstract

The microbiology of caves is an important topic for better understanding subsurface biosphere diversity. The diversity and taxonomic composition of bacterial communities associated with cave walls of the Oylat Cave was studied first time by molecular cloning based on Sanger/pyrosequencing approach. Results showed an average of 1,822 operational taxonomic units per sample. Clones analyzed from Oylat Cave were found to belong to 10 common phyla within the domain Bacteria. *Proteobacteria* dominated the phyla, followed by *Actinobacteria, Acidobacteria* and *Nitrospirae.* Shannon diversity index was between to 3.76 and 5.35. The robust analysis conducted for this study demonstrated high bacterial diversity on cave rock wall surfaces.

Key words: bacterial diversity, Oylat Cave, Sanger/pyrosequencing, subsurface biosphere

Introduction

Diversity, composition, and functional roles of microorganisms in the habitable extreme environments have been intensively studied in the last decades. Caves are one of the habitable extreme habitats, and microorganisms have been determined in these ecosystems based on independent culture techniques in late 1990s such as in Nullarbor Cave, Australia (Holmes *et al*., 2001); Wind Cave, United States (Chelius and Moore, 2004); Niu Cave, China (Zhou *et al*., 2007); Altamira Cave, Italy (Portillo *et al*., 2009); Kartcher Caverns, United States (Ortiz *et al*., 2014).

Turkey has a great geothermal potential due to a high degree of orogenic, magmatic, and volcanic activity, as part of the Alpine-Himalayan orogenic belt (Ketin, 1966). Due to active faults and volcanism, there are more than 600 terrestrial hydrothermal vents, mainly in the Aegean Region, Northwest, Middle Anatolia, East and Southeast Anatolia regions. In addition, there are more than 20,000 caves in Turkey; however, a limited study has been conducted on the microbial diversity in these cave systems.

Oylat Cave (39°56'36"N, 29°35'26"E) is located 17 km south of the town Inegol, which is 80 km southeast of Bursa city, Turkey. Oylat Cave has formed due to karstification within the Permian-Triassic recrystallized limestone, develops along two fault zones in WNW-ESE and NE-SW directions. In the three parts creating the cave, debris and carbonate sediments have accumulated. In the first part, there are debris stores including pebble stone, sand stone and silt stone and carbonate things including stalactite, stalagmite, column, cave pearls. In the second part, carbonate formations are present and these are cave breaches, stalactite, stalagmite, column, macaroni structures, curtain stalactites, cave pearls, giant stalactites pools. In the third part, mudstone, siltstone, sand stone and thick cave breaches are present (Atabey *et al*., 2002).

The purpose of this work was to determine the bacterial diversity and composition at Oylat Cave (Bursa, Turkey) using a combined Sanger and 454 pyrosequencing approaches. Even though, previous studies have documented distinct bacterial communities in limestone caves in the world (Barton *et al*., 2007; Legatzki *et al*., 2012), the current work is first effort to document bacterial diversity and taxonomic composition for Oylat Cave (Bursa, Turkey).

Experimental

Materials and Methods

Sample collection and DNA extraction. Cave wall samples were collected using sterile spatula and stored in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI, USA) in September 2013, Oylat Cave (Fig. 1 and 2).

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After collection, the samples were frozen on dry ice on site, and stored at –20°C upon return to the laboratory (Groth *et al.*, 1999). Environmental DNA was extracted from samples using Fast DNA Spin kit for soil (MP biomedicals, Solon, OH USA).

Clone library construction and Sanger sequence analysis. Total genomic DNA was used as a template for 16S rRNA PCR amplification using bacteria 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') universal primers (Weisburg *et al.*, 1991). Each 20 µl reaction mixture contained: l µl environmental DNA template, 2.25 mM $MgCl_2$, 2 µl GeneAmp 10X PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 100 µM dNTPs (Sigma-Aldrich, Saint Louis, MO, USA), 0.2 µM each primer, 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling was as follows: initial denaturation 5 min at 94°C, 25 cycles of 94°C for 1 min, hybridization at 50°C for 25 s and elongation at 72°C for 2 min followed by a final elongation at 72°C for 20 min. PCR products were purified using a QIAquick kit (QIAGEN, Valencia, CA, USA), and were cloned into *Escherichia coli* hosts using the TOPO TA Cloning kit with the pCR 2.1 Vector (Invitrogen Corporation, Carlsbad, CA, USA). Plasmid DNA was extracted and purified using the Ultra Clean Standard Mini Plasmid Prep Kit (MoBio Laboratories). Cloning products were sequenced by TUBITAK MAM DNA Services Facility at Gebze, Turkey, using standard M13 primers.

Partial sequences were assembled with CodonCode Aligner v.1.2.4 (CodonCode, USA) and manually checked. Assembled sequences were checked for chimera by Bellerophon server (Huber *et al.*, 2004) and Chimera_Check v 2.7 (Cole *et al.*, 2005). Sample sequences were aligned by BioEdit (Ibis Biosciences, Carlsbad, CA, USA). Phylogenetic analysis was performed in PAUP (Sinauer Associates, Sunderland, MA) using parsimony, neighbor-joining, and maximum likelihood analyses. The 16S rRNA gene sequences were submitted to the NCBI Gen Bank database under accession numbers JQ065958-JQ065959 and JQ219081-JQ219137.

454 pyrosequencing and sequence analysis. For the pyrosequencing, the V6 region of the 16S rRNA gene was amplified using PCR with a bacterial primer set 967f (5'-MWACGC GAR GAA CCT TAC C-3') and 1070r (5'-AGC TGACGA CAR CCA T-3') (Baker *et al.*, 2003). A single-step 30 cycle PCR using HotStar Taq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 40 s, and 68°C for 40 s and final extension at 68°C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). The samples were sequenced with a Roche FLX 454 (Roche, Mannheim, Germany) in a commercial facility (Research and Testing Laboratories, Lubbock, TX).

Raw results 454 pyrosequencing analyzed with combining the QIIME (Quantitative Insights into Microbial Ecology), Mothur and RDP (ribosomal database project) programs as described in Nakayama *et al.* (2013). The obtained sequence data were depleted of barcodes and primers then short sequences < 200 bp are removed by using the QIIME split library. Sequences with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6 bp removed. Sequences were then denoised and chimeras removed using the Chimera.uchime program in Mothur (http://www.mothur.org/wiki/Download_mothur) (Edgar *et al.*, 2010; Wright *et al.*, 2012). Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity) by using the pick_otus_through_otu_table.py script of QIIME. OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis and Hugenholtz, 2006) and compiled into each taxonomic level into both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative (proportion) percentage of sequences within each sample that map to the designated (Cole *et al.*, 2005; Wang *et al.*, 2007) taxonomic classification using the RDP Classifier (http://rdp.cme.msu.edu). Sequences were deposited in the NCBI Sequence Read Archive under accession number PRJNA258221.

SEM (Scanning electron microscopy) with EDS. Scanning electron microscopy was used to examine the morphological structure of the samples in high resolution. The sample was fixed in glutaraldehyde for 3–4 hours. Fixed samples were then dried, mounted and coated with gold (Borsodi *et al.*, 2012). The samples were examined using a Jeol JSM – 7001FA scanning electron microscope at an accelerating voltage of 15 kV. For the microanalyses of the chemical element composition of the samples were accomplished by EDS using.

Results

Field observation and SEM investigations. Samples were collected from three locations inside the cave, fossil part, section II and section III (Fig. 1 and 2). Scanning electron microscopy (SEM) images revealed that sample were composed of a mass of calcite crystals (Fig. 3a). Solid samples on walls at Oylat Cave is also presented in Fig. 3b and Fig. 3c. Elemental analysis of solids at fossil part sample indicates that the highest

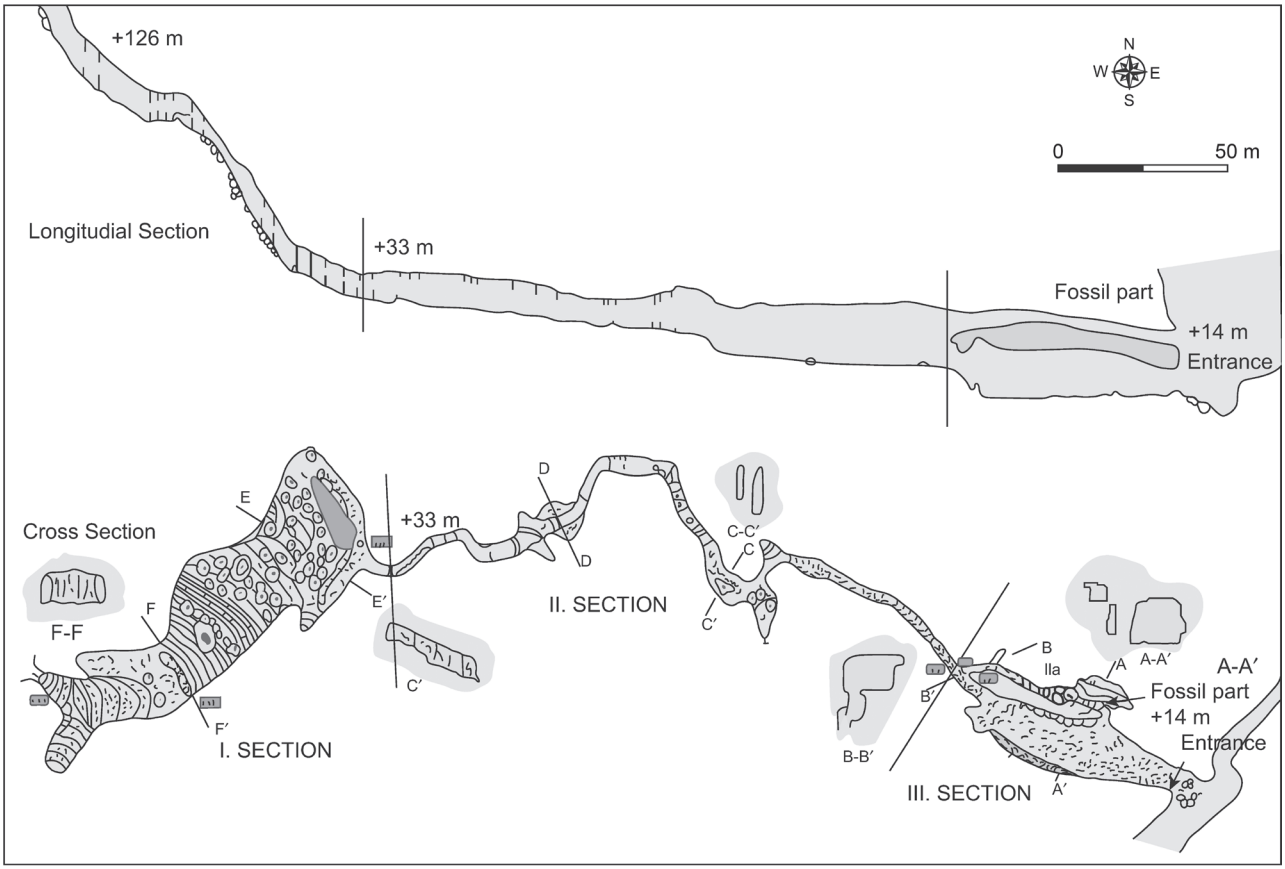

Fig. 1. Map of Oylat Cave (Atabey *et al.*, 2002).

(wt. %) of calcium (Fig. 3d). The highest elemental wt. % iron is observed in wall rock sample collected from the section III (Fig. 3e).

16 S rRNA gene library. To investigate the microbial diversity in Oylat Cave (Bursa, Turkey), a 16S rRNA clone library for bacteria was constructed and 87 clones were randomly selected and analyzed. The majority of the sequences identified from the clone libraries belonged to the *Proteobacteria* taxonomic division, specifically the *Gammaproteobacteria*, *Betaproteobacteria,* and *Alphaproteobacteria*, as well as from other bacterial divisions, including *Actinobacterium*, *Acidobacterium*, *Bacteroides*, *Gemmatimonodates*, *Verrucomicrobia*, *Firmucutes*, *Chloroflexi Planctomycetes* and *Nitrospirae* divisions. As shown in Figures 4a, uncultured *Solitalea* sp. clone OYLT, a *Bacteriodetes* that clustered in a lineage with a *Solitalea korensis* strain had 99% similarity. Putative *Actinobacteria*, clustered with a clone from Pajsarjeva jama cave, Slovenia (FJ535083) and Oylat sample clone have ≥ 99 similarity. For *Chloroflexi* phyla, uncultured chloroflexi bacterium clone OYLT show similarity ≥ 95 similarity with uncultured chloroflexi bacterium clone (FJ535096). The unique OTUs belonged to a diverse group of phyla including *Acidobacteria*, *Nitrospirae*, *Planctomycetes*, *Firmicutes* and *Gemmatimonadetes* (Fig. 4a). *Proteobacteria* clones were phylogenetically associated with 3 classes of *Proteobacteria* with similarities between 85%–95 (Fig. 4b). *Alphaproteobacteria* clone is uncultured *Sphingomonas* sp. OYLT clone was 95% similar to uncultured *Sphingomonas* sp. (KC172197), a soil heterotrophic bacterium. The majority of the Sanger OTUs analyzed were associated with heterotrophic bacteria in the phylogenetic analysis (Fig. 4b).

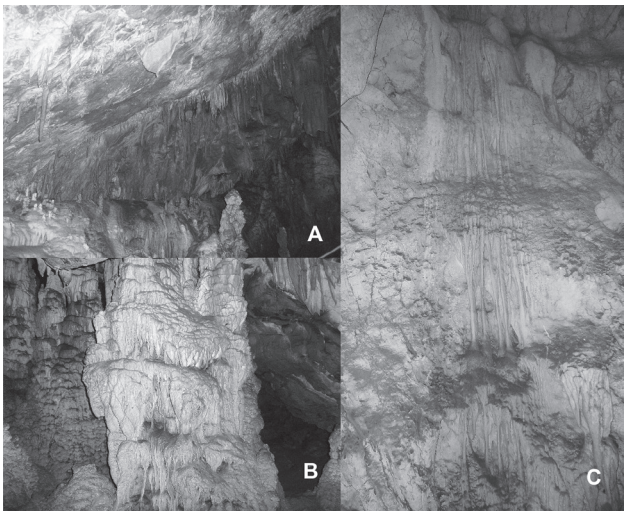

Fig. 2. Sampling locations at Oylat Cave (2a, 2b, 2c).

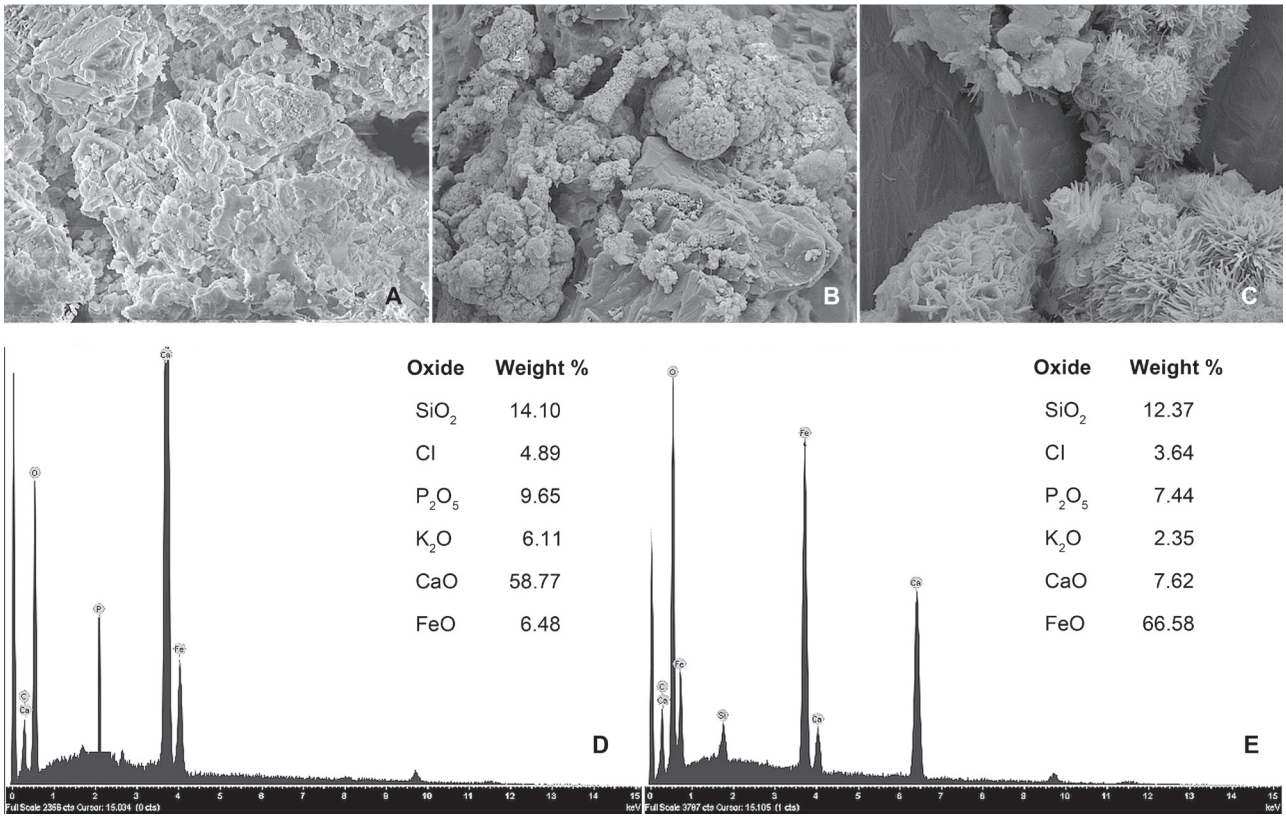

Fig. 3. Scanning electron micrograph of from Oylat Cave and EDS results (3a, 3b, 3c, 3d, 3e).

Diversity and taxonomic analysis of bacterial communities with 454 Pyrotag. The 454-pyrosequencing of three samples generated a total of 152,629 sequence reads after quality filtering and contaminant removal, representing 85% of the original dataset. Sample library size ranged from 3276 to 20,653 sequence reads. Alpha diversity index at Oylat Cave samples (Table I) presented the high biodiversity in all diversity metrics. Number of OTU was determined between 1428 and 2457. Chao1 was calculated as 2,507 and 3,944. The range in the Shannon diversity index for the each sample was 3.76 to 5.35.

Bacterial community structures were determined for each sample based on analysis of 454 pyrosequencing. A total of 10 bacterial phyla were identified from Oylat Cave. The bacterial communities were dominated by *Proteobacteria*, with abundances ranging from 42–63% (Fig. 5a) for all sampling sites. The *Proteobacteria* was composed of *Deltaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* in the samples (Fig. 5b). Second dominated phyla is *Actinobacteria* in the (OYLT1) sample, followed by *Acidobacteria*, *Nitrospirae*, *Firmicutes*, *Bacteriodetes*, *Planctomycetes*, *Gemmatimonadete*, *Verrucomicrobia* and *Chloroflexi* (Fig. 5a). In the other sample is (OYLT2), *Proteobacteria* (48%) dominated, followed by *Acidobacteria*, *Actinobacteria*, *Planctomycetes* and *Nitrospirae*. Third sample is (OYLT3) dominated *Proteobacteria* (63%), followed by *Actinobacteria*, *Nitrospirae*., *Acidobacteria*, *Planctomycetes*, and *Firmicutes.*

Table I
Summary of 454 – pyrotaq OTUs and diversity and richness estimates

Sample	Number of OTUs	Chao1	ACE	Shannon
OYLT1	1,583	2,670	3,429	4.58
OYLT2	2,457	3,944	4,328	5.35
OYLT3	1,428	2,507	3,317	3.76

[a] Calculated using ACE richness estimator

Discussion

The deep subsurface remains one of the least explored microbial habitats on Earth, despite the increasing number of investigations in the past decade. The microbiology of caves is an important topic for better understanding subsurface biosphere diversity.

Limestone caves, such as Altamira Cave in Italy (Portillo *et al.*, 2009), the Niu Cave in China (Zhou *et al*., 2007), the Pajsarjeva jama cave in Slovenia (Pasic *et al*., 2010), Kartchner Caverns in the United States of America (Ortiz *et al*., 2012), and Jinjia Cave in western Loess Plateau of China (Wu et al., 2015) have been

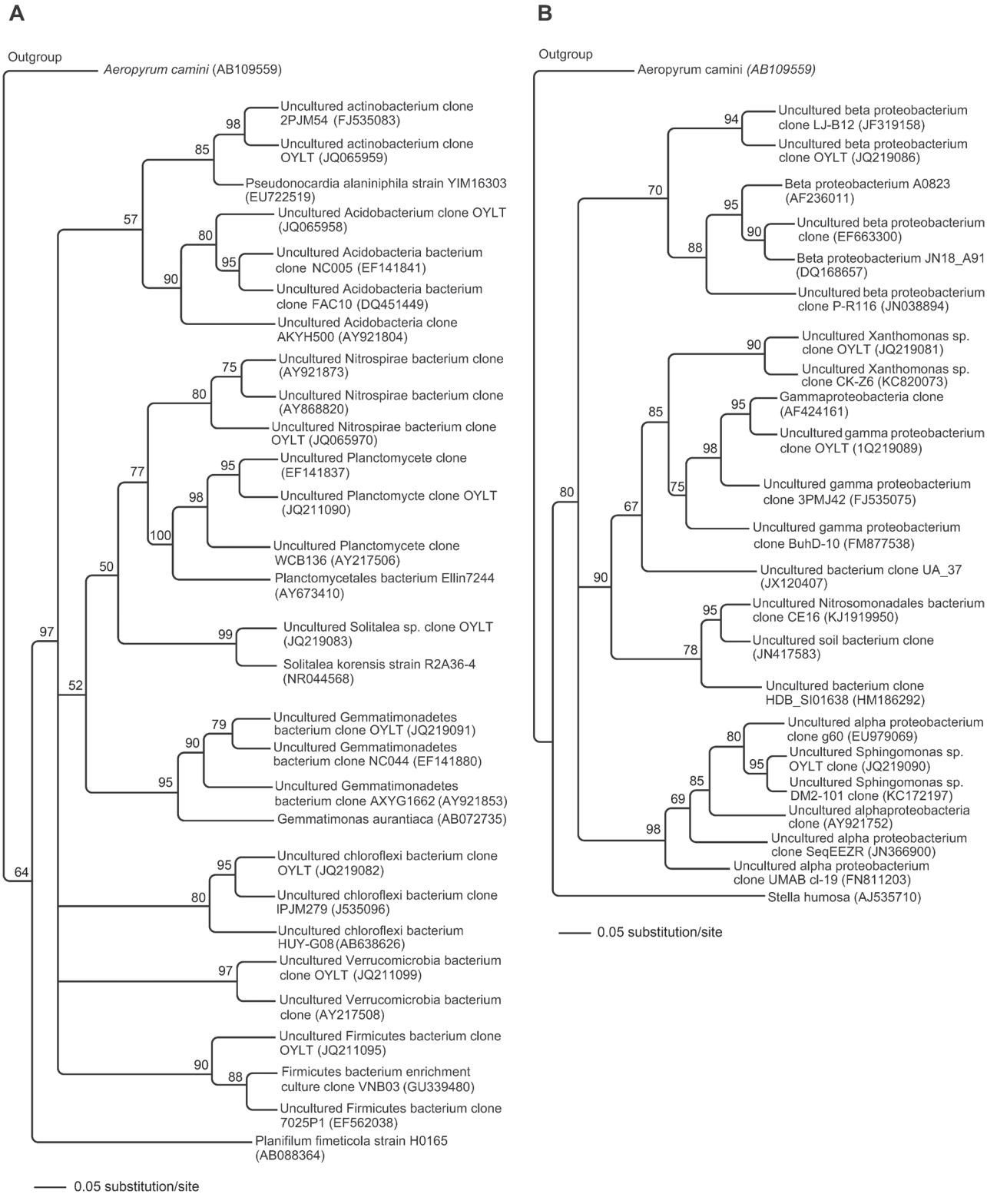

Fig. 4. Maximum parsimony phylogenetic inference of 16S rRNA sequences obtained from environmental clones at Oylat Cave.
4a. Phylogenetic tree of gene sequences associated with non-*Proteobacteria* phyla;
4b. Phylogenetic tree of gene sequences associated with *Proteobacteria* classes.

microbiologically and geochemically studied in last decade. In this study, bacterial phylogenetic diversity and composition observed in Oylat Cave (Bursa, Turkey) using Sanger and 454 pyrosequencing.

In other limestone cave studies, *Proteobacteria* was identified as the dominant phylum, with *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* classes being most common (Schabereiter-Gurtner

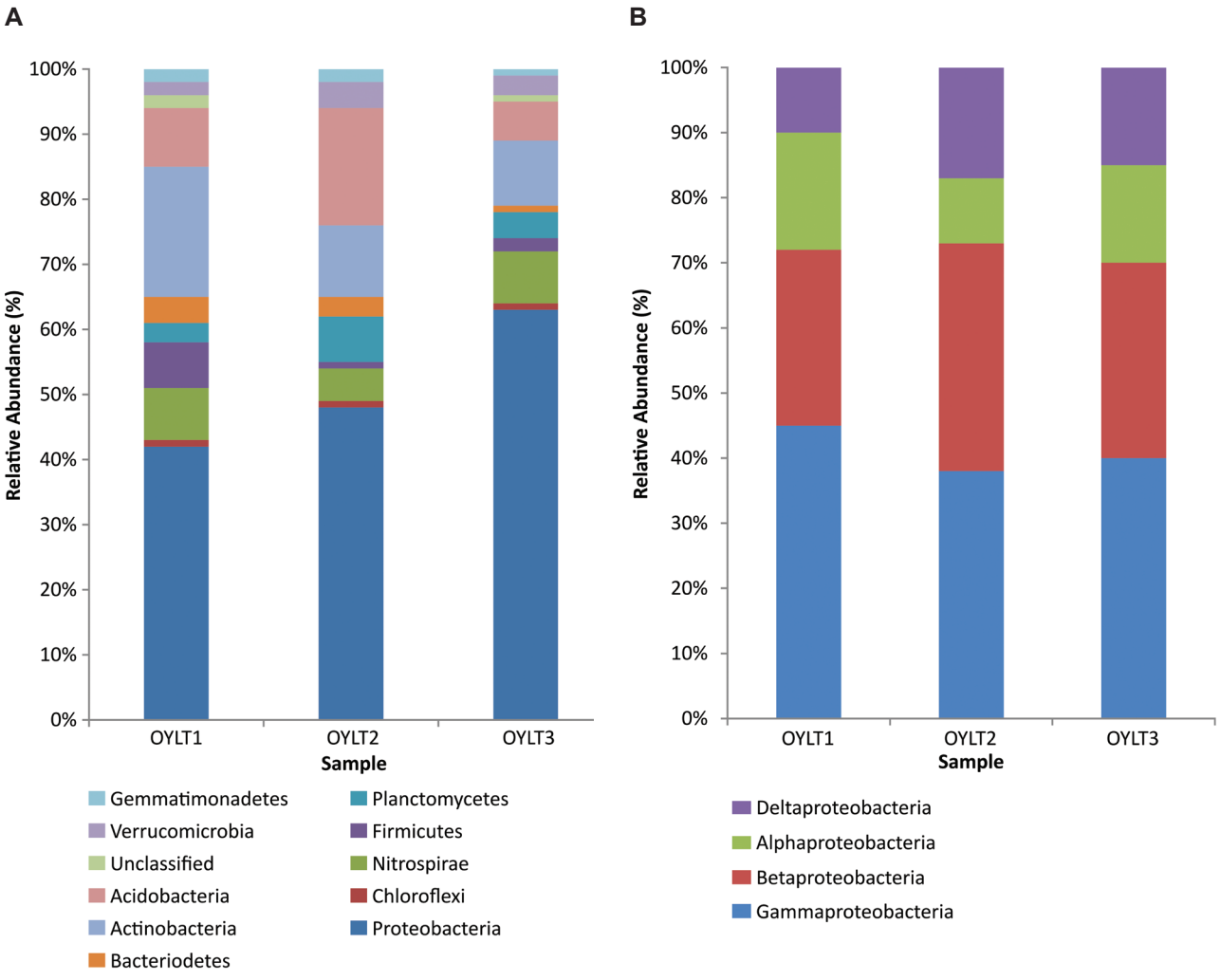

Fig. 5. Bacterial community composition of 454 sequence libraries.
5a. Distribution of dominant phyla in Oylat Cave samples: OYLT1 represent fossil part; OYLT2 represent Section II; OYLT3 represent Section III;
5b. Distribution of *Proteobacteria* (classes) in 454 samples.

et al., 2004; Barton *et al.*, 2006; Ortiz *et al.*, 2012). Similarly, the results of this study revealed that *Proteobacteria* phyla is dominant, also *Gammaproteobacteria* are dominant classes in the structure of community. Both the Sanger and pyrotag OTUs for *Gammaproteobacteria* related to sequences from different habitats (soil, hot spring, sewage, ground water). From comparing the distributions of *Proteobacteria* phyla, a core limestone microbiome becomes apparent.

Actinobacteria and *Acidobacteria* were second and third dominating phylogenetic groups in Oylat Cave respectively. Barton *et al.* (2007) found *Actinobacteria* to be the dominant phylum, representing 60% of the bacterial community of an oligotrophic limestone rock surface, in Carlsbad Caverns, New Mexico. *Actinobacteria* members are typical heterotrophs, actively participate in the carbon cycle by degradation of organic wastes (Ivanova *et al.*, 2013). Also, they have functional role on the biomineralization in the cave ecosystems (Zhou *et al.*, 2007). *Acidobacteria* found abundantly in several karstic cave environments, however their functional role unknown at present (Pasic *et al.*, 2010).

Nitrospirae members are likely to occur in different cave ecosystems. For instance, *Nitrospirae* clones observed from the extremely acidic Frasassi Cave (Macalady *et al.*, 2006). Members of *Nitrospirae* also observed in the limestone caves, Pajsarjeva jama Cave and Tito Bustillo Cave (Schabereiter-Gurtner *et al.*, 2002; Pasic *et al.*, 2010). *Nitrospirae* were identified as the fourth most abundant phylum in the overall Oylat Cave microbial community. *Nitrospirae members* especially *Nitrospirales* order's play role on nitrogen cycling such as nitrite oxidation in cave environment (Ortiz *et al.*, 2012).

Additional components of the bacterial cave wall microbial community belonged to the phyla *Firmicutes*, *Planctomycetes*, *Bacteroidetes*, *Verrucomicrobia*, *Gemmatimonadetes* and *Chloroflexi,* respectively in Oylat

Cave. These phyla reported from different limestone caves worldwide (Schabereiter-Gurtner *et al*., 2004; Zhou *et al*., 2007; Barton *et al*., 2007; Pasic *et al*., 2010; Ortiz *et al*., 2012; Wu *et al*., 2015).).

In conclusion, this diversity and taxonomic analysis conducted in Oylat Cave provides key information about these microbial communities. Both the Sanger and pyrosequencing clone library, robust analyses results to provide clues to potential energy sources in the cave, such as carbon and nitrogen cycling. In future studies, focus on functional metagenomics effort in Oylat Cave to determine the presence of clones closely associated with bacteria that have carbon- and nitrogen-fixing capabilities.

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