The Primary Results of Analyses on The Archaeal and Bacterial Diversity of Active Cave Environments Settled in Limestones at Southern Turkey

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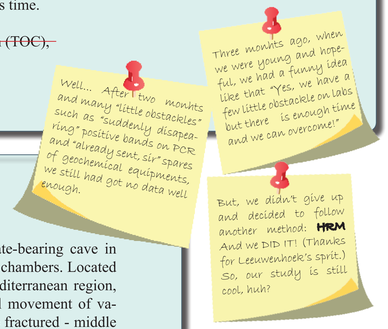
ABSTRACT

The microbial diversity of cave sediments which are obtained from three different caves named Insuyu, Balatini and Altınbesik located at Southern Turkey has been investigated using molecular methods for biomineralization. The total number of 22 samples were taken in duplicates from the critical zones of the caves at where the water activity is observed all year round. Microbial communities were monitored by 16S rRNA gene based PCR-DGGE (Polymerase Chain Reaction - Denaturating Gradient Gel Electrophoresis) methodology. DNA were extracted from the samples by The PowerSoil® DNA Isolation Kit (MO BIO Laboratories inc., CA) with the modifications on the producer's protocol. The synthetic DNA molecule poly-dIdC was used to increase the yield of DNA extraction-PCR amplification via blocking the reaction between CaCO3 and DNA molecules. Thereafter samples were amplified by using both Archaeal and Bacterial universal primers.

Subsequently, archaeal and bacterial diversities in cave sediments, were investigated to be able to compare with respect to their similarities by using ~~DGGE. DGGE patterns were analysed with BioNumerics software 5.1. Similarity matrix and dendograms of the DGGE profiles were generated based on the Dice correlation coefficient (band-based) and unweighted pair-group method with arithmetic mean (UPGMA). The structural diversity of the microbial community was examined by the Shannon index of general diversity (H).~~ HRM

High-resolution melting (HRM) analysis is a rapid and robust molecular tool for microbial community fingerprinting. Kim and Lee (2014) has recently reported that HRM produced robust community clustering and ordination results comparable to the results from the commonly used denaturing gradient gel electrophoresis (DGGE) performed in parallel. This method transforms melting peak plots of community DNA samples generated by HRM analysis to molecular fingerprints and estimates the relationships between the communities based on the fingerprints. In this study, we used HRM for bacterial and archaeal community fingerprinting for its high-throughput capacity and short analysis time.

Similtaneously, geochemical analyses of the sediment samples were performed within the scope of this study. ~~Total organic carbon (TOC), x-ray diffraction spectroscopy (XRD)~~ and x-ray fluorescence spectroscopy (XRF) analysis of sediments were also implemented.
The extensive results will be obtained at the next stages of the study currently carried on.



RESEARCH AREA

İnsuyu Cave

Insuyu Cave is an extensive, Calsium Carbonate-bearing cave in shape bed rock dominated passages with several chambers. Located in Burdur Province, one of the arid part of Mediterranean region, Insuyu has been formed by the continious tidal movement of vadose water at the area throught Upper Createase fractured - middle Maestrihtiyen Pelagic Limestones which are characterized with high permeability. In addition to dynamic water table, also, the synthetic Riedel faults contributed to speleogenesis are the main reason of unconsolidated and fractured texture of the cave.

Ofiolitic melange unit within the relict characteristic is dominant as bedrock of the cave formed in border of the two different formation which are the Quaternary aluvial sediments and the Eocene Limestones. Insuyu Cave, which extends towards northeast direction with a total length of 8.350 meters, has two different characteristics as the part in strongly eroded, neritic limestones that formation created long, squeeze tunnels like a cobweb and the part in the same limestone but a bit denser. Although there are huge collapses in the further section, there are no small branches and galleries. Due to huge usage of underground water and increasing number of artesian wells around Burdur lowlands in the last years, water level in Insuyu Cave decreased as much as 7 meters. This situation resulted by the complete loss of two lakes but, on the other hand, gives an opportunity to search new galleries which were previously underwater.

Altınbeşik-Balatini Caves

The field within the extensive underground water system which Altınbeşik and Balatini caves are the parts of it is represented with the Createase and Jura-Cretase Limestones and Paleocene-Eocene Flysch deposits. In addition to the litology of the units, the factors as tectonism, karstication and fluvial erosion derived from Manavgat River are also effective on morphology of the field.

Altınbeşik Cave is located in Cretase massive Limestones. The cave has a lake 125 meters long at the enterance within the depth 15 meters. The lake ends with a huge travertine wall which is 44 meters high and tree main branches follow this wall, along ... meters. The cave abounding in travertine speleothems discharges enormous amount of water at spring and winter periods.

MATERIAL & METHOD

Sampling

The total number of 22 samples were taken in duplicates from the critical zones of the caves at where the water activity is observed all year round.
Averagely, samples each one of 2.5 gr to 5 gr were placed sterile polipropane bottles and tubes. Humidity and temperature were measured at all of the sampling point by ExStik2 (Extech Instruments, NH 03063 U.S.A)

Extraction

DNA were extracted from the samples by The PowerSoil® DNA Isolation Kit (MO BIO Laboratories inc., CA) with the modifications on the producer's protocol. At the first expriments, the results from agarose gel electrophoresis represented that the possible inhibition during DNA extraction. Ca^{++} was determined as inhibition factor by XRF analysis and the alternating copolymer, poly-deoxyinosinic-deoxycytidylic acid sodium salt, was used to increase the yield of DNA extraction via blocking the reaction between CaCO3 and DNA molecules.

PCR amplification and Agarose Gel

Microbial communities were monitored by 16S rRNA gene based PCR (Polymerase Chain Reaction) methodology. Thereafter samples were amplified by using both Archaeal and Bacterial universal primers which are 7F-1384R/344FGC-522R and PA-PH/VFGC-VR. Each PCR mixture was prepared in a final volume of 25 µL: 1 µL of template DNA, 0,5 µL of each primer (final concentration, 100 nM) and Taq Polimerease, 1 µL of dNTP, 19 µL of PCR-grade water, and 2,5 µm L of buffer. After, then, the products are monitored under trans-UV.

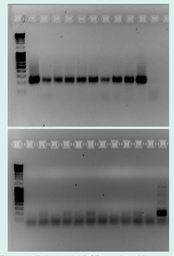


Figure1: Eubacterial PCR results of Insuyu Cave
Figure2: Archeal PCR results of Insuyu Cave

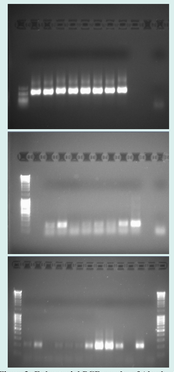
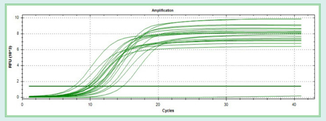


Figure3: Eubacterial PCR results of Altınbesik-Balatini Caves
Figure4: Archeal PCR results of Altınbesik Cave
Figure5: Archeal PCR Balatini Cave (7 to 10 holes)

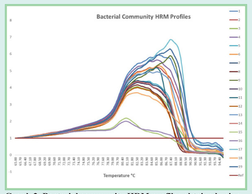
HRM Analysis

Subsequently, archaeal and bacterial diversities in cave sediments, were investigated to be able to compare with respect to their similarities by using HRM. Nested qPCR approach was used to amplify microbial rDNAs (Kolukirik et al. 2011). The first round qPCRs were carried out using Arch46f-Arch1017r and Bact8f-Bact1541r primer sets targeting archaeal and bacterial rRNA coding genes respectively. Arch344f-Univ522r and Bact342f-Bact534r primer sets were used for the second round PCRs. The following thermal cycling conditions were applied for all of the qPCRs: 3 mins at 95 °C; 40 cycles of 20 secs at 95 °C, 20 secs at 53 °C and 30 secs at 72 °C. BiospeedyTM HRM Master Mix (Bioeksen Ar-Ge Teknolojileri, Turkey) and Biorad CFX connect instrument was used for all reactions. The reactions contained 1.5mM MgCl2, 0.2mM dNTP mix, 1x Reaction Buffer, 0.1U Fast Start Proof Reading Recombinant Taq DNA Polymerase, 1x EvaGreen, 5ng/µL DNA template and 0.5µM of each primer. To ensure and detect whether if the expected product is amplified during q-PCR and for HRM analysis, melting curve analyses were applied between 60°C-95°C at a florescence reading rate of 0.1°C/acquisition. HRM profiles were obtained as described by Reja et al. (2010). Microbial community profile dendrograms were obtained using Minitab 17 software based on the similarities between the HRM profiles. Bivariate correlation analyses between the microbial and chemical characteristics of the samples were performed using MINITAB 17. The correlations were evaluated using Pearson's method. Statistical significance was taken as $p < 0.05$. Principal component analysis (PCA) ordinations were calculated in MINITAB 17. The variance (eigenvalue) associated with a principal component versus the number of the component was plotted to judge the relative magnitude of the eigenvalues. The first principal components (PC1s) for the archaeal and bacterial HRM profiles have the eigenvalue 43.629 and 55.587 that accounts for 92.8% and 94.8% of the total variance, respectively. In other words, most of the HRM fingerprinting data structure was captured in PC1. For this reason, PC1 was correlated to the other variables for determination of the factors that affect the microbial community structures.

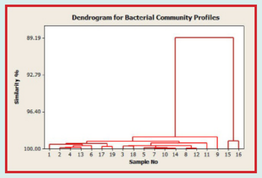
Results of Bacterial Community Analyses-HRM



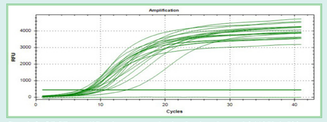
Graph 1. QPCR amplification curves obtained using the Bact342f-Bact534r primer set



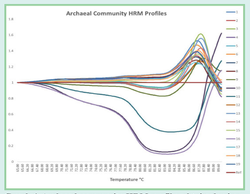
Graph 2. Bacterial community HRM profiles obtained using the Bact342f-Bact534r primer set



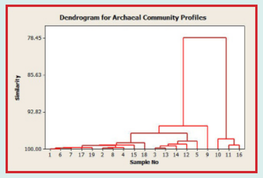
Results of Archaeal Community Analyses-HRM



Graph 3. QPCR amplification curves obtained using the Arch344f-Univ522r primer set



Graph 4. Archaeal community HRM profiles obtained using the Arch344f-Univ522r primer set



Statistical Analyses

PC1 of archaeal and bacterial community profiles and geochemical data (as ppm values) from XRF analysis of the samples were given in Table 1. The correlation analyses between the geochemical parameters and the microbial compositions revealed that there is a statistically significant correlation between K levels and archaeal community profiles of the samples (r=0.489, p=0.034). This implies that K might be a limiting nutrient determining archaeal community structures in the samples. We will further test this finding along with the relative ratio of K to the total organic carbon, nitrogen and phosphorus contents.

PC1 Archaea	PC1 Bacteria	Ca	K	Si	S
-0.261	-0.23	275403.375	12721.06	30293.59	1017.266
-0.255	-0.241	416344.156	1510.937	2824.437	956.747
-0.246	-0.244	390911.188	2618.455	8359.169	835.918
-0.252	-0.239	402271.813	2506.894	9081.168	460.101
-0.235	-0.244	470865.438	1196.345	2920.437	1683.758
-0.26	-0.244	462559.75	0	0	1572.95
-0.259	-0.242	356105.688	7637.211	24123.94	1730.035
-0.251	-0.234	454591	719.648	0	1151.091
-0.153	-0.227	81610.977	20367.32	113929.4	0
0.084	-0.241	119560.839	1486342	12158.3	268.078
0.128	-0.241	407925.938	1052.79	1782.111	1610.193
-0.225	-0.236	302574.893	5907.333	50750.25	1524.223
-0.239	-0.243	280161.938	5469.753	46990.97	1411.318
-0.226	-0.243	288566.796	5633.846	48400.7	1453.658
-0.261	-0.148	423159.094	2468.727	16589.3	1024.482
0.114	-0.168	431622.276	2518.102	16921.09	1044.972
-0.26	-0.207	373136.719	2594.799	25702.02	430.421
-0.256	-0.243	430641.188	10000.88	9088.791	6817.903
-0.262	-0.217	380599.453	2646.695	26216.06	439.0294

In accordance with the significant similarity of the microbial profiles between the samples taken from different caves, it could be considered that the conclusive factor of microbial community profiles of caves is not directly releated with the geographical regions. The results of cluster analysis show that the main factor is intenal conditions and inorganic resources.

To be continue...