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ORIGINAL RESEARCH PAPER

Bacterial diversity determination using culture-dependent and culture-independent methods

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ABSTRACT: Mud volcanoes are taken into consideration by geologists and oil industry experts have given their association with oil and gas reserves and methane greenhouse gas production in hydrosphere and atmosphere. Gomishan mud volcano phenomenon in the southeastern edge of the Caspian Sea, given its oil and gas resources, has been studied by some geologists in terms of geology and tectonics but not in terms of microbiology. Accordingly, it seems necessary to study this phenomenon from the perspective of microbiology in order to identify prokaryotes living in this area. Prokaryotes diversity in Mud volcano has been studied by cultivation techniques, fluorescence in situ hybridization, and denaturing gradient gel electrophoresis of PCR-amplified fragments of 16S rRNA genes. Total cell abundance in the mud volcano from 1×10^{1}-6×10^{1}per milliliter was determined by 4', 6-diamidino-2-phenylindole direct count. The detectable proportion of Archaea to Bacteria in the community by FISH was one to five. High viable counts ($1-3\times10^{6}$) were obtained in culture media. A total of 122 isolates were obtained, 46 colonies were selected based on primarily morphological and physiological traits, and their 16S rRNA sequences were determined. The isolated genera included *Halomonas* (20%), *Arthrobacter* (5%), *Kocuria* (5%), *Thalassobacillus* (5%), *Marinobacter* (20%), *Paracoccus* (5%), *Roseovarius* (5%), *Jeotgalicoccus* (5%), *Bacillus* (15%), and *Staphylococcus* (15%). Regarding DGGE analysis, selected bands were obtained from the gels, reamplified and sequenced. Overall, 75% of the bacterial sequences were related to *Rahnella* and 25% related to *Serratia*.

KEYWORDS: *Denaturing gradient gel electrophoresis (DGGE); Deoxyribonucleic acid (DNA); 4, 6-diamidino-2-phenylindole (DAPI); Extraction; Fluorescence in situ hybridization (FISH); Mud volcano; Phylogenetic tree; Polymerase chain reaction (PCR).*

INTRODUCTION

Prokaryotes constitute a large part of the biosphere and are less noticable than other groups. They are responsible for a lot of processes in the life cycle and are influential on vitality of all living groups.

The new gene pools introduce biodiversity or identification the variety of microbial community which is the basis for biotechnological application (Griffiths, *et al*., 2016). Diversity of microorganisms without the use of a variety of different ways to view microorganisms and identify the differences between them is not possible. Identifying the differences between the morphology, physiology and phylogenetics of microorganisms is possible only with the help of methods and powerful tools. These methods can be

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divided into two parts, culture-dependent and culture-independent methods (Sathyanarayana Reddy, *et al.,* 2016). Mud volcanoes are natural and native ecosystems which have been taken into consideration by geologists in the last few decades due to their relationship with oil and gas reserves (Kopf, 2002). Mud volcanoes are cone-shaped and consist of sediment and hot water which find their way as loose, watery mud flow from the crater of the cone to the surface and they spew into the air as a fountain because of containing boiling hot water and volcanin gases. Mud volcanoes are known as the geological source of atmosphere carbon such as methane gas. They are geological structures that emit gas, water, and clay compounds to the surface of the land or the sea floor (Dimitrov, 2003; Bharti and Niyogi, 2015). Since mud volcanoes are mainly created in the hydrocarbon-bearing sediments ranging from seabed or aquatic environment sidelines to faults and subductions, they are served as an appropriate environment for microorganisms which convert methane to carbon dioxide through anaerobic oxidation (Zemskaya, *et al.,* 2010). On the other hand, overproduction of carbon dioxide disrupts the food chain and increases the emission of greenhouse gases, adversely affecting the environment through destruction of ecology and aquatic habitat at sea due to methane gas emission and increased greenhouse gases will result human societies damage. Mud volcanoes emit gases such as methane, ethane, propane, butane, pentane, and other non-greenhouse gases such as CO_2, N, H_2S, Ar, and He. In fact, these are sites for accumulation of bacteria which use methane or hydrogen sulfide (Suess, *et al.,* 1999).

Gomishan, in the southeast region of the Caspian Sea, Iran, is located at 7 km northeast of Gomishan City. It is a large puddle of water around 25 m. diameter with a very soft mud edge and gas bubbles in the middle. The study has been carried out in the Gomishan mud volcano, in Iran in 2013 since the area has not yet been studied microbiologically. The research focuses on bacteria diversity in the area and aims to a) describe its bacterial community using both cultivation and culture-independent approaches and b) compare the results obtained using both approaches, c) discuss in more detail the specific characteristics of mud volcano community.

MATERIAL AND METHODS

Sampling and Chemical characteristics

Samples from Gomishan mud volcano were taken in August 2013. The mud volcano is located in Golestan Province, 7 km from Gomishan City with geographic coordinates of 54˚6’36”E, 37°12’26”N (Fig. 1). It is a large puddle of water of around 25 m. diameter and very soft mud edge, with gas bubbles in

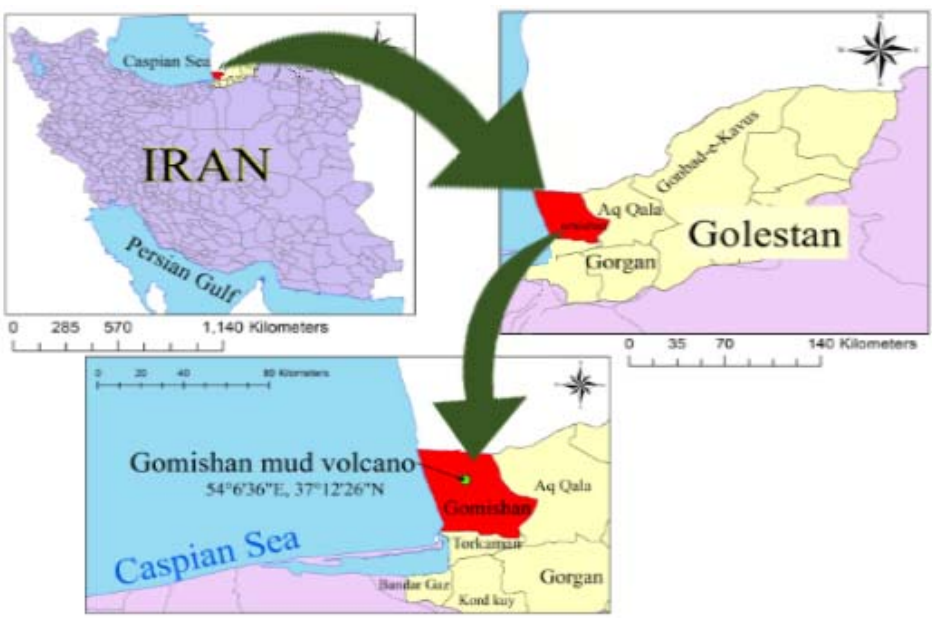

Fig. 1: The Gomishan mud volcano study area

the middle. The mud volcano can be seen circular in the middle of the plain. It is surrounded by salt water with traces of oil in some parts. Compounds are usually exited as severe eruptions discontinuously and irregularly after moments of calmness and quietness, which are associated with the smell of gas. A total of 20 samples were collected from different places of the mud volcano. Geographic coordinates, pH, and temperature were measured in the area. The samples were quickly transported to the laboratory in 500 µL sterile polypropylene bottles (20 bottles), kept in darkness, and stored at 4 °C for microbial testing and -80 °C for molecular analysis.

The salinity and pH of the samples were measured after transferring to the laboratory by a pH meter/conductivity (Mettler), and the amounts of cations (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, Fe^{2+}) were determined respectively at 422.7, 285.2, 766.5, 589.0, 248.3 nm wavelenghth by Atomic absorption spectroscopy (Model 3030, Perkin-Elmer, USA), and anions (HCO3-, Cl^{-}) were determined by titration.

Fleuorecence in situ hybridization (FISH)

The Arch 915 (Stahl and Amann, 1991) and EUB338 (Amann, *et al.,* 1990) probes were used to identify members of the Archaea domain and the Bacteria domain, respectively. The sample was first put on a Millipore filter (0.22µm). Next, 100 µl of 2% Tween 80 was added to 50 g of mud. Then sample was mixed well and kept at 4˚C for one night. Following that, 2 ml of formaldehyde were poured in a sterile Falcon and the supernatant of the sample prepared the day before was added to Falcon to raise the volume to 50 ml. The falcon was kept at 4˚C for one night, and then 10 ml of phosphate-buffered saline (PBS) was added to the sample, and the sample was filtered on a Millipore filter (0.22 µm) (In this stage, the filters can be stored for up to one month in a freezer at -20˚C). One hundred µl of lysozyme (10 mg/g) was added onto the filter, the filter was kept at 37˚C for 60 minutes, and then it was then washed with distilled water. From this stage on, the test was continued in darkness. Eighteen µl of hybrid buffer was poured on the filter, 2µl of probe was added to it, and the mixture was gently and carefully made uniform. The filter was then kept in a moist Falcon at 46˚C for 2 hours and, following that, it was placed in the washing solution for 15 minutes. This washing process was carried out twice, and the filter was then stained with DAPI. After that, it was first washed in water, then in 70% and 96% alcohol, air-dried, and fixed to a slide. The slides were kept in darkness at 4˚C until the test time. After the slides were prepared in the method mentioned above, the samples were examined by utilizing a fluorescence microscope (Olympus DX 59, Japan) and suitable filters.

Isolation of microorganisms, culture media and growth conditions

In order to determine the microbial diversity of the mud volcano, mud samples were inoculated in the prepared culture medium immediately after transfer to the laboratory (within 24 h.). Inoculation was performed through serial dilutions. Bacteria were cultured on the modified Heterotrophic Growth medium with 0.5 % and 5 % salt exposed to air and were incubated at 34 °C.

The composition of the modified Heterotrophic Growth medium contained (g/L): 10 mL sodium pyruvate, 10 mL $MgSO_4.7H_2O$, 10 mL $CaCl_2.6H_2O$, 2 g yeast extract, 5 g peptone, 0.5 g ammonium sulfate, and 15 g agar. NaCl was added to the culture media concentrations: 5 g/L to one and 50 g/L to the other. The medium pH was adjusted to 8 with 1M NaOH prior to autoclaving. Colony formation was investigated after 2, 3, 7, and more than 7 days during which the plates were examined, then the colonies were counted, and the obtained isolates were transferred to new media. To ensure the purity of the isolates, they were cultured sequentially. Purity of the colonies was confirmed by using the Gram staining based on Hacker's method (Gerhardt, *et al.,* 1994) and optical microscopy with lens 100.

Morphological, physiological, and biochemical features

In this step, all isolates were examined in terms of shape and colony characteristics, gram reaction, microscopic shape and arrangement of cells, shape and location of spores, and catalase and oxidase reactions. The antimicrobial susceptibility test was carried out according to the disk diffusion method at a concentration of 30 µg/disc chloramphenicol and 5 µg/disc erythromycin to distinguish the archaea and bacteria (Elevi and Oren , 2008).

DNA extraction and Amplification of 16S rRNA gene

Genomic deoxyribonucleic acid (DNA) was extracted through the Marmur manual method of DNA extraction in which the prepared biomass was dissolved in 200 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) in 1.5 mL vials. Then, 20 mL of 10 mg/mL lysozyme solution was added and incubated at 37 °C for 30 min. After adding 25 mL SDS 10 %, the vial was gently inverted and kept at 37 °C for 60 min. Ninety microliters of 5 M NaCl was added to the solution and the vial was inverted gently several times and then incubated in 65 °C water bath for 20 min. A volume of chloroform was added to the solution and the vials were gently stirred and then centrifuged at 11500 rpm for 15 minutes. The upper aqueous phase was transferred to a sterile tube. In order to increase the accuracy, step five was repeated twice. In order to precipitate DNA, cold isopropanol was added to 0.6 of the volume of the previous step solution and kept at -20 °C overnight. After 15 minutes of centrifugation at 11500 rpm and discarding the supernatant, the sediment was washed with 70 % alcohol. The final precipitate was dried at room temperature, dissolved in 50 mL TE buffer after drying, and stored at -20 °C. 16S rRNA gene of the selected strains was amplified with the universal reverse and forward primers of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for bacteria (Lane, *et al.,* 1985), respectively. PCR was performed in a final volume of 50 µL including the following components: 1.3 µL of each primers, 0.6 µL dNTP (10 Mm), 3.4 µL PCR buffer, 1 µL $MgCl_2$ (50 Mm), 2 µL DNA template, 2 µL enzymes, and 41.5 µL PCR-specific water. The PCR amplification program for this gene consisted of 95 °C for 5 min, continued by 30 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, and finally 72 °C for 7 min.

The method introduced by Yang *et al.* (2012) was used to obtain the environmental DNA in this research. A mixture of 100 mg mud, and 600µl of TENP (100 mM NaCl, 20 Mm EDTA, 50 mM Tris, 0.01 g/ml PVP, pH 10) buffer was prepared. The sample was stirred at 2000 rpm for 2 minutes. The Eppendorf microcentrifuge tube was centrifuged at 12000 rpm for 2 minutes, and the obtained precipitate was washed with PBS buffer. The sample was mixed with 400µl of DNA extraction buffer (1.5 M NaCl, 100 mM EDTA, 100 mM sodium phosphate, 100 mM Tris, 1% CTAB, pH 8.0), 8mg/g of skim milk powder, and 0.07g of glass beads in a 1.5ml Eppendorf microcentrifuge tube. 1µl of proteinase K was added to tube followed by shaking at 2000 rpm for 2 minutes. The sample was kept in an incubator shaker at 37˚C for 30 minutes, then 400µl of 20% (w/v) SDS was then added to it, and the Eppendorf microcentrifuge tube was placed in a water bath at 65˚C for 2 hours. After centrifugation at 11000 rpm, the aqueous phase was transferred to a new tube and mixed with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v), and the tube was centrifuged at 11000 rpm for 15 minutes. The aqueous phase was transferred to a new sterile tube. 0.6 volume of cold isopropanol was added to the solution obtained from the previous stage and the tube was placed at 4˚C for one night. It was then centrifuged at 11000 rpm for 15 minutes, the supernatant was discarded, and the obtained precipitate was washed with 70% ethanol. This stage was repeated twice. The supernatant was discarded, the final precipitate was dried at room temperature, 40 µl of TE buffer was added to the precipitate, and the sample was kept at -20˚C (Yang, *et al.,* 2012).

For DGGE analyses, the extracted DNA are separated from each other employing the GC clamp 357F (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCGCCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCMTTTGAGTTT) primers and using differences in migration patterns in gel due to differences in their resistance to the denaturing material which itself is caused by different base compositions (Muyzer and Smalla, 1999). The PCR protocol was used for amplification of this gene before DGGE included preliminary denaturation at 95˚C for 5 minutes, followed by 35 cycles of denaturation, each at 94˚C for 45 seconds; annealing at 52˚C for 45 seconds, and the initial 45-second extension at 72˚C followed by final extension at 72˚C for 7 min. DGGE was applied by means of DCode system (Mutlu, *et al.,* 2008).

Sequencing and sequence analysis

Sequencing was conducted at Macrogen (Seoul, South Korea). Isolated 16S rRNA genes and DGGE bands were sequenced directly. The obtained sequences were edited and prepared for analysis in the

databases by using Bioedit and Chromas. Sequences obtained from environmental studies or known species were similar to with each strain identified through search in Gene Bank (BLAST) and EzTaxon (Chun, *et al.,* 2007). Phylogenetic analysis of the selected isolates and similar strains was carried out by using ClustalX, Bioedit, and Mega 6. The phylogenetic tree of these strains was plotted by applying Maximum Likelyhood and Neibour Joining methods (Tamura, *et al.,* 2013).

RESULTS AND DISCUSSION

Identification of new genetic resources has become a permanent necessity of biotechnology, given the growing need for new metabolites in industry, medicine, and the environment. Nature is the richest source of new genes and biodiversity of prokaryotes forms; a large part of the biodiversity in the biosphere, while prokaryotes have received less attention than other life groups. Prokaryotes exclusively perform the processes of life cycle of materials that influence preservation of all living groups (Torsvik, *et al.,* 2002). Prokaryotes have remained largely unknown so far (Achtman and Wagner, 2008), and this shows the importance of conducting research related to identification and biodiversity of these microorganisms. Investigation of biodiversity in native and unique ecosystems in each region is valuable due to various adaptations that take place during evolution. Use of the available methods for distinguishing microorganisms has shown that only one percent of all microorganisms can grow and be isolated in pure culture (Torsvik, *et al.,* 1990). Mud volcanoes are one of the areas that many scientific efforts have been performed over the past three decades to raise the knowledge on formation and dynamics. Along with geological, geophysical, and biogeochemical studies, several microbial and biological analyses were also performed on the structure of mud volcanoes (Milkov, *et al.,* 2004). Iran has a great diversity of mud volcanoes whose microbial population needs to be elucidated. Gomishan mud volcano is located in the southeast region of the Caspian Sea, Iran, with geographic coordinates of 54˚6'36''E, 37°12'26''N. Media with different percentage of salt and long incubation were used for isolation of species with the highest amount culturable bacteria in mud volcano. The initial isolation was performed after 48 hours and plates were stored for several months to slow down growing bacteria. Competition between them was prevented through direct inoculation in solid media. Based on some common procedures, environmental samples are usually cultured in enriched liquid media at first and then are transferred to solid culture media to isolate the strains; however, this can disrupt the actual composition of the population. By direct inoculation of environment sample into solid culture media competition-induced demographic change is avoided. Finally, the long incubation period creates the possibility of colony formation for the potential slow-growing types.

Sample characteristic, total DAPI cell count and FISH analysis

The temperature of mud was about 38 °C with a pH of 8. The measured salinity was about 34 ppm. The amount of cations and anions of the mud was depicted in the Table 1. Sodium and chloride were the highest and iron was the lowest ion of the mud. CFU counts (live counts) in the culture method using a medium containing 0.5 % and 5 % salt were (on average) 3×10^6 and 1×10^6, respectively, which is the same as the numbers found in other environments having similar conditions.The number of viable cells counted in the mud volcano in the DAPI method was about $1\text{-}6\times 10^1$ cells/mL of muddy water. The FISH test showed the estimated ratio of archaea to bacteria was about 1:5. Moreover, the Bacteria domain accounted for about 85 % of the prokaryote population in the region.

About 0.007% of the observed microorganisms applying the direct count method for enumerating bacteria with DAPI were obtained in the culture method and approximately 0.006% in the direct count method with FISH test. Although the FISH

Table 1: Chemical characteristics of the sampled from the Gomishan mud volcano

Elements	Na^+	Mg^{2+}	K^+	Ca^{2+}	Fe^{2+}	Cl^-	$HCO3^-$
Sample (g/L)	18.4	0.18	0.14	0.23	0.06	15.97	0.183

test is widely used and has many applications, yet it has limitations. Enumeration of bacteria by employing FISH test and using general-purpose probes rarely yields the same results as those obtained in using other methods such as labeling nucleic acids. The probability of finding the target cell in FISH test covers the wide range of zero to 100%. Ecosystem type substantially influences determination of the percentage of cells using the EUB 338 probe. In samples taken from swamps, the number of bacterial cells is very small compared to other samples including those taken from seawater, which indicates it is very difficult to extract and distinguish bacterial cells in samples taken from swamps (Bouvier and Del Giorgio, 2003; Xie, *et al.,* 2016). Since the sample in this research was taken from a swamp, and because it was very sticky due to the presence of clay minerals, it was not an exception to the general rule.

Therefore, extraction of microorganisms was not very successful. In studies conducted on other mud volcanoes, bacterial enumeration using FISH test and DAPI was not done and, hence, there is no information in this regard. The study of slides and the 3 % KOH reaction showed that the frequency of isolates were 46.7 % Gram-negative rod-like, 29.5 % Gram-positive cocci, and 23.8 % gram-positive rod-like. Regarding the catalase reaction, most isolated bacteria were oxidase-positive and catalase-positive. The isolates were then divided into several groups based on phenotypic characteristics, and those with similar characteristics were placed in the groups. Finally, 42 diverse strains were selected randomly from these groups for phylogenetic studies. In order to differentiate bacteria and archaea, the antimicrobial susceptibility the test was performed by using chloramphenicol and erythromycin. All strains were sensitive to these antibiotics. Consequently, all isolated strains were bacteria.

Phylogenetic analysis

Among the 122 isolates purified in the culture method, 42 strains with the gene sequence of 16 Sr RNA were studied (Fig. 2).

Results indicated that they belonged to 4 Classes and 20 different genera. Eight strains (40%) were of the *Firmicutes* and eight (40%) of the *Gammaproteobacteria*. Furthermore, one strain (10%) belonged to the *Alphaproteobacteria* and one strain (10%) to the *Actinobacteria*. The phylogenetic tree for each class was drawn by using the Maximum Likelihood method. The position of the studied strains on the tree, are presented in Figs. 3 and 4. In the present research, 25 halotolerant bacteria and 17 moderately halophilic bacteria were isolated. In the moderately halophilic strains, the maximum frequencies were those of the two genera *Marinobacter* and *Halomonas*, which have a marine origin and are strongly halotolerant; this result is anticipated by the marine source of this mud volcano with a short distance to the sea. The majority of the salt tolerant strains were relatives of the *Bacillus* and *Staphylococcus* genera. However, in research Liu et al. carried out in 2011on a mud volcano in southern Taiwan, Gram-positive *Clostridium* spp. and gram-negative *Schewanella* spp. were isolated. Moreover, Heller *et al.,* 2011 separated *Clostridium thiosulfatireducens* from the Salse di Nirano mud volcano in Italy. On the Gharniaregh mud volcano, *Bacillus* were isolated by Diansani *et al.,* in 2012. Differences in isolates may be due to the structure of the culture medium used for isolating bacteria, the natural distribution of microorganisms in the environment, and the ecological characteristics of the region (Alain and Querellou , 2009). The probability of increases in the existing diversity with each increase in the number of times samples are taken also influences the differences in the types and numbers of microorganisms isolated in any research (Hughes, *et al.,* 2001).

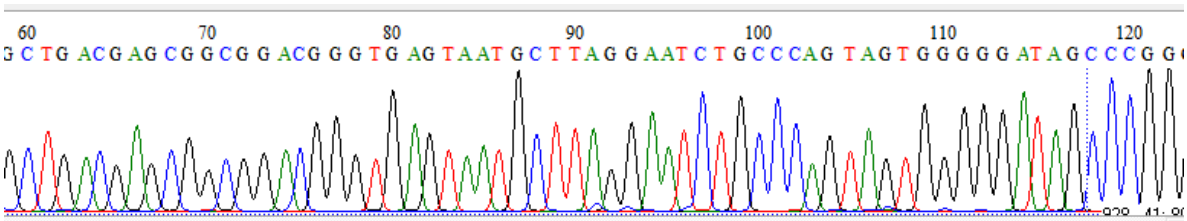

Fig. 2: The gene sequence of 16 S rRNA

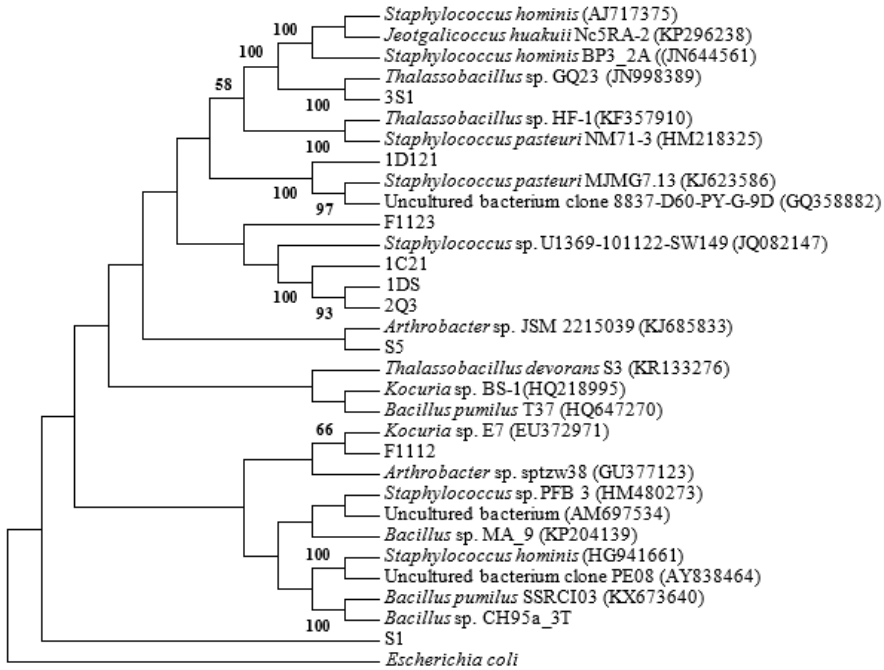

Fig. 3: The phylogenetic tree using Maximum likelihood of the S16 rRNA related to Gram-positive bacteria found in this research using the culture method and the bootstrap coefficient of 100. Bootstrap coefficients below 50 have been omitted. The registration numbers of the sequences in the NCBI Gene database are presented in parentheses. The *Escherichia coli* sequence has been included as the out-group.

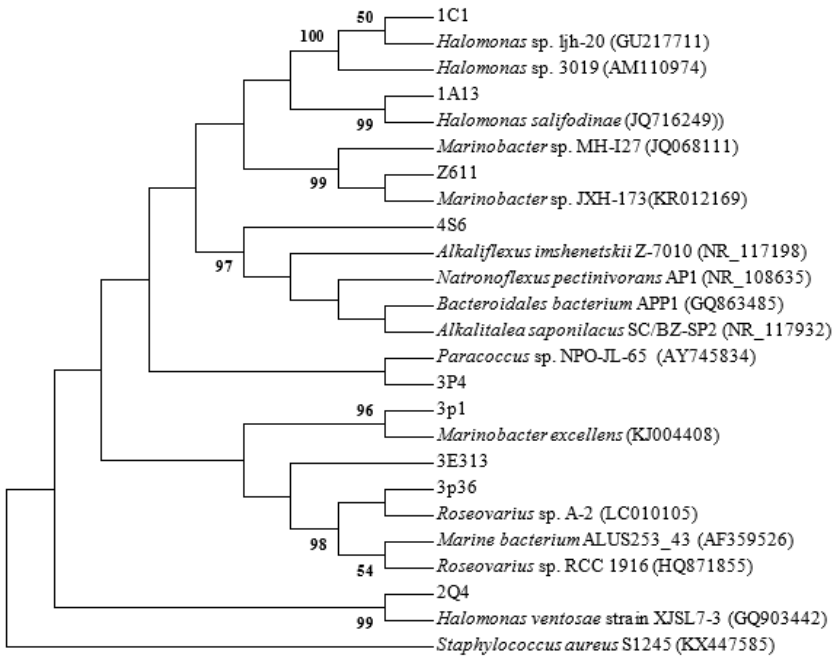

Fig. 4: The phylogenetic tree using Maximum likelihood of the16S rRNA related to Gram-negative bacteria found in this research using the culture method and the bootstrap coefficient of 100. Bootstrap coefficients below 50 have been omitted. The registration numbers of the sequences in the NCBI Gene Database are presented in parentheses. The *Staphylococcus aureus* sequence has been included as the out-group.

In this research, which was conducted based on sequencing the 16S rRNA gene in selected strains, the strains belonged to four Classes (*Alphaproteobacteria, Gammaproteobacteria*, *Actinobacteria*, and *Firmicutes*) and 10 different genera. The phylogenetic tree for each Class was drawn by using the Maximum Likelihood method, and the position of the studied strains on the tree, are presented in Figures 3 and 4. In addition to their low population density in the environment, the small number of isolated bacteria could be due to the fact that optimum conditions were not used in isolating the bacteria. Among the sequenced isolates, 8 (40%) belonged to the *Firmicutes* Class, 8 (40%) to the *Gammaproteobacteria* 1 (10%) to the *Alphaproteobacteria*, and 1 (10%) to the *Actinobacteria*. These results are close to that of Diansani *et al.* (2011) on the Gharniaregh mud volcano. In their research, similar to the present study, most strains belonged to the phylum *Firmicutes*. Since Gomishan and the Gharniaregh mud volcanoes are about 50 km apart to some extent it confirms the close structural relationship between the two, it was not unexpected to find comparable results. Furthermore, research on the sediments of the Amsterdam mud volcano in the Mediterranean Sea showed that both *Archaea* and *Bacteria* were found close to each other in the sediment layers. Most of the bacterial groups belonged to *Proteobacteria*, especially to *Gammaproteobacteria* (Pachiadaki, *et al.,* 2010). Their results are comparable with this research. 50% of the strains were of *Proteobacteria* and 40% of which belonged to *Gammaproteobacteria*. In Gulf of Mexico the Pseudomonas genus of *Gammaproteobacteria* formed 61% of the isolated bacterial samples (Lanoil, *et al.,* 2001). This is probably because *Gammaproteobacteria* are the most common culturable bacteria in soil and water and there have been simple culture conditions on the other *Proteobacteria* are diverse and are mainly obtained under natural conditions. Here, the similarity of each strain to the nearest known microorganism recorded in Gen-Bank was 98-100%, and the highest percentage of similarity was observed in the genera *Marinobacter*, *Paracoccus*, and *Bacillus*. The degree of simile exhibited by most of the strains to the closest known microorganisms varied from 98% to 100 %. This meant that the purpose of the research, which was to identify new species, was largely unfulfilled. The similarity of each strain to the nearest known microorganism recorded in GenBank was 89-98%, and the highest percentage of similarity was seen in the genera *Pseudomonas*, *Ralstonia*, and *Clostridium* was found in marine sediments (Zemskaya, *et al.,* 2010). Eleven of the 42 sequenced strains, which were those of the various species in the genera *Marinobacter*, *Paracoccus*, *Staphylococcus*, *Bacillus*, *Jeotgalicoccus*, and *Geofilum*, had 100% alikeness in the sequence of 16S rRNA, which gives them importance with respect to being presented to the National Bank of Microorganisms as native identified microorganisms. Moreover, the degree of similarity in two strains belonging to the *Roseovirus* genus was 97-98.4%, and they may be placed in new species when DNA-DNA hybridization with closely related species is done and phenotypic traits are studied. Twenty-nine strains had 98.5-99.9% similarity in sequencing (Stackebrandt and Ebers, 2006).

In the culture-independent method (DGGE), the most frequent sequences were those of the *Rahnella* genus so that 75% of the obtained sequences were members of this genus in the bacterial genomic library, with the genus *Serratia* accounting for the remaining 25% (Fig. 5). Isolation of *Serratia* has not been referred to in a similar research conducted on mud volcanoes. On the Wushanding mud volcano in Taiwan isolated *Clostridium* and *Pseudomonas* and most of the species were of the nonculturable type (Chen, 2008).

In a research in Russia, 260 clones in 25 phenotypes with high bacterial diversity were isolated from the Malenky mud volcano (Russia's Lake Baikal) including bacterial colonies *Deltaproteobacteria*, *Gammaproteobacteria*, *Chloroflexi*, and OP11and *Bacteroidetes, Cytophaga, Flexibacter* and *archaea* colonies such as *Crenarchaeota* and *Euryarchaeota* (Zemskaya, *et al.*, 2010).

To determine general structure and prokaryotic diversity of mud volcano in the Xinjiang region of China, researchers isolated 100 bacterial and 100 *archaea* colonies from their sediments. The bacteria belonged to 11different phylotypes, and were finally classified into the three clones of

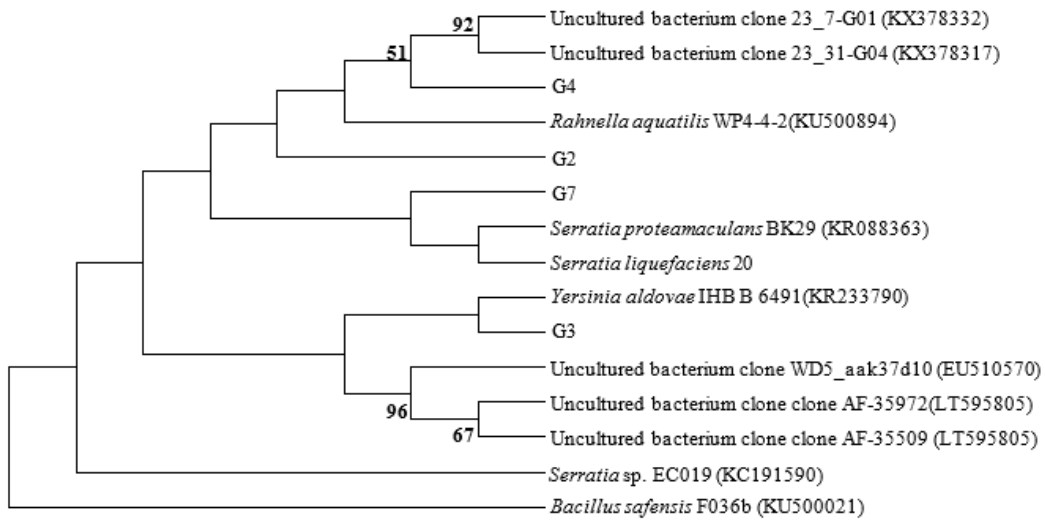

Fig. 5: The phylogenetic tree using Maximum likelihood of the16S rRNA related to DGGE found in this research using the culture method and the bootstrap coefficient of 100. Bootstrap coefficients below 50 have been omitted. The registration numbers of the sequences in the NCBI Gene Database are presented in parentheses. The *Bacillus safensis* sequence has been included as the out-group.

Proteobacteria, Actinobacteria, and *Fusobacteria*. It was also noticed that the *Deltaproteobacteria* of the *Proteobacteria* group were the most frequently found and the preferred group compared to the other four *Proteobacteria* clones and were related to the *Desulfuromonadales* order. The *Archaea* phylotypes were of nonculturable species and mostly of the *Methanosarcinales* and *Halobacteriales* orders of the *Euryarchaeota* group and could tolerate methane hydrate or alkaline sediments. Since Xinjiang region is a nearly dry region of low water resources not close to any ocean, it is different from mud volcanoes such as San Biagio Belpasso and Paclele Mici in Romania that seem to be of marine origin. Therefore, most microorganisms found in this region are probably native of it (Yang, *et al.*, 2012). The observed differences between the various mud volcanoes and the one we studied can be attributed to the various characteristics of the different habitats. The various weather and biological characteristics of each region and its substrate type influence the extent and type of the biodiversity that is expected to be found in it so that biodiversity of each habitat is considered one of its unique characteristics. Therefore, the importance of conducting research on new environments does not decrease with increases in the number of studied environments (Alain and Querellou, 2009).

Comparison of the culture method and DGGE in microbial diversity of the Gomishan mud volcano

Results obtained from each of the methods have special limitations and bias that depend on the innate characteristics of the method and the way it is implemented. In the culture method, besides the main limitation (access to only about 1% of the existing microorganisms), the culture media, and the culturing method do not cover a wide scope due to financial and time limitations. Therefore, this method only allows growth of a special group of microorganisms and results obtained do not represent the real situation in the whole population. In the molecular method, no culture media are used and access to the environmental microorganisms is possible without the need to grow them under artificial laboratory conditions. Nevertheless, another barrier, access to genetic materials, and the ability to amplify the desired gene in this method, creates limitations, and not all the microorganisms in the region respond the same way to the DNA extraction method that is employed. The mild extraction method prevents DNA extraction from a section of the existing microorganisms and these microorganisms have no place in the identified microbial population. Similarly, the harsh extraction methods break and omit DNA of some microorganisms the genomes of which are

more easily extracted. In the amplification stage also, although universal primers are used, yet the characteristics of the primers and the employed amplification method are not the same for all strains and some are amplified more easily and form a larger percentage of the identified population. In the present research, in order to reduce bias in obtained result a culture medium similar to the environment in which the microorganisms lived was used in the culture method, and various methods of amplifying the environmental DNA were employed. According to the obtained results, it is not impossible to culture the identified genera in the culture-independent method. However, because of the various above-mentioned reasons, including various biases and needs of the culture media, they could not be isolated on plates. Moreover, the dominant genera in the two methods were completely different. The *Marinobacter* and *Halomonas* with 20% were the dominant genera in the culture dependent method, while these genera were not isolated in the culture-independent method. In fact, there was no overlap between the results obtained in the culture-dependent and culture independent methods in this research. This can be attributed to the very small number of sequenced isolates using the DGGE method for sequence determination. In studying microbial communities, increasing the number of samples is not as effective as it is in studying plant and animal communities and cannot be generalized. However, results that are more acceptable are obtained when the number of samples is increased. Results of the present research show that the best results in the introduction of biodiversity in a region are achieved by employing polyphasic methods including simultaneous use of conventional culture-dependent methods and molecular methods. Use of various tools provides us with a more correct view regarding biodiversity of microorganisms of a given region.

CONCLUSION

The study on the prokaryotic biodiversity in the Gomishan mud volcano was performed through using the culture method and denaturing gradient gel electrophoresis. Moreover, direct enumeration of microorganisms in the mud volcano was done and the *archaea*: *bacteria* ratio was determined by using the DAPI and FISH staining methods. Based on results obtained in this research, the Gomishan mud volcano is similar to other mud volcanoes in the world with respect to *Gamma*proteobacteria. Considering the bacteria isolated in the culture-dependent and culture-independent methods in this research did not overlap. It seems polyphasic methods including simultaneous use of both the culture method and the molecular method yield more complete results regarding biodiversity in swamp environments such as mud volcanoes.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript.

ABBREVIATIONS

DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DAPI	4, 6-diamidino-2-phenylindole
FISH	Fluorescence in situ hybridization
PCR	Polymerase chain reaction
NCBI	National center for biotechnology information
SDS	Sodium dodecyl sulfate
CFU	Colony forming unit

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