

Full Length Research Paper

## Biodiversity of uncultured bacteria in hypersaline lakes, Siwa oasis, Egypt, as determined by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rRNA gene phylotypes

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Bacterial populations within hypersaline lakes often exhibit uncultured unique species. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), followed by sequencing of 16S rRNA gene approach was applied in order to explore, for the first time, the bacterial communities within the hypersaline Lakes, Aghormy, Zeiton and Maraqi, Siwa Oasis, Egypt. The DGGE profile displayed 12 phylotypes of 16S rRNA gene, representing the total species richness within the three studied lakes. The phylotypes were varied among lakes, but were restricted to two phylogenetic groups, *Bacteroidetes*, which occurred in all lakes, and *Alphaproteobacteria*, which showed abundance in only Lakes Zeiton and Maraqi. Single spirochete-like phylotype characterized the Lake Aghormy. A phylotype, which was recorded in both of Aghormy and Zeiton, represented a first recorded of *Lewinella agarilytica* in hypersaline inland lakes. Sequence homology results suggested novel indigenous bacterial phylotypes.

**Key words:** Siwa lakes, halophilic bacteria, 16S rRNA gene, molecular diversity.

### INTRODUCTION

Hypersaline environments are distributed worldwide, and saturated salt inland lakes are among the most physical and chemical demanding habitats on earth. Few groups of organisms survive in them; rare eukaryotes and many halophilic prokaryotic cells are capable of living in saltern

crystallizers (DasSarma and DasSarma, 2012).

Halophiles are salt-loving organisms, capable of growing in salt concentrations more than 150 g/L (15% w/v, 2.5 M), which flourish in saline environments (Ollivier et al., 1994). Halophiles are generally more specialized

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organisms and are found in all three domains of life, that is, bacteria, archaea and eukaryotes.

Halophilic bacteria are abundant in many phylogenetic subgroups, most of which belong to Halomonadaceae, a family of Proteobacteria (Oren, 2002). Also, hypersaline environments are dominated by members of Bacteroidetes, often *Salinibacter ruber* (Anton et al., 2002). These bacterial species are aerobic, anaerobic, chemoheterotrophic, photoheterotrophic, and/or photoautotrophic. They are ubiquitously found in lakes and coastal areas worldwide, e.g., in the Dead Sea (Wei et al., 2015), Lake Urmia, Iran (Mehrshad et al., 2015), solar salterns in Tunisia (Boujelben et al., 2012), the Tuzkoy salt mine, Turkey (Mutlu and GUVen, 2015), Rambla Salada, Murcia, Spain (Luque et al., 2014), the Great Salt Lake, Utah, USA (Tazi et al., 2014), Dagong Brine Well, China (Xiang et al., 2008) and the Capuchin Catacombs, Italy (Pinar et al., 2014).

Halophilic bacteria have been studied by cultured- and uncultured-depending techniques, with sequence-based approaches for phylogenetic and taxonomic classification (DasSarma and DasSarma, 2012). For uncultured halophiles, sequencing and phylogenetic analyses of 16S ribosomal ribonucleic acid (rRNA) gene expanded the breadth of diversity of unknown species, among halophiles (Edbeib et al., 2016). Hence, application of these molecular approaches to study hypersaline environments have presented unique prokaryotic communities and found to be a valuable source of novel prokaryotic diversity.

The lakes in Siwa Oasis, western desert, Egypt, are athalasoaline environments, characterized by high salt concentrations and unique water parameters (Abd El-Karim and Goher, 2016). The low raining and high evaporation rates within those lakes favored the development of hypersaline environments. The Siwa lakes have been studied from the views of physicochemical characteristics and as a source of some environmental biotechnological applications (Abd El-Karim, 2016, Abd El-Karim and Goher, 2016, Mahmoud and Abd El-Karim, 2016). However, the uncultured bacterial communities within those lakes remained yet to be uncovered. In this study, application of bacterial 16S rRNA gene analysis, through PCR/DGGE and sequence approach, was done in order to explore the halophylic bacterial richness within those lakes.

## MATERIALS AND METHODS

### Sampling

Sampling was done from three Lakes, Aghormy, Zeiton and Maraqui, Siwa Oasis, Egypt (Figure 1). The lakes are characterized by water depths between 0.5 m, Aghormy and Zeiton, and 0.7 m, Lake Maraqui. The water pH values were 7.83, 7.36 and 7.61, for Aghormy, Zeiton and Maraqui, respectively. The most feature which

characterizes those lakes is hypersalinity, where the recoded total dissolved solids, TDS, mainly sodium chloride, were 220.03, 239.29 and 280.45 gL<sup>-1</sup> in the water of Aghormy, Zeiton and Maraqui, respectively. One liter of surface water microbial mat was collected from the margins of each of the three studied lakes. The collected microbial mats were treated with sterile TE buffer, 50 mM EDTA, 50 mM Tris-HCl, pH 7.6 to chelate as much as possible of cations in the collected saline water, and consequently, protect DNA from degradation inside the microbial cell. The treated microbial mates were stored at 4°C for molecular analyses.

### DNA extractions and PCR amplifications

Bulk microbial DNAs were extracted from treated microbial mats, using DNA isolation kit (MO BIO Laboratories, 12888-50, Carlsbad, CA), according to the manufacture's protocol with modifications of Elsaied (2013). Three replicates of 50 ml of each collected microbial mat were centrifuged for 5 min, with speed 10000 rpm, at 4°C to concentrate the microbes. Chemical lysis was conducted, using mixture of 5 M guanidine thiocyanate (Sigma) and 10% sodium dodecyl sulfate, SDS, and incubated at 75°C for 20 min with strong shaking. The homogenates were briefly centrifuged for 10 min at 10000 rpm and the supernatant lysates were removed in clean sterilized propylene tubes. The bulk DNA, of the three replicates for each lake water sample, was purified from the crude lysates, using Sephadex columns and finally combined with each other. The purified DNAs, extracted from all lake water samples, were run on 0.9% agarose gel electrophoresis, followed by staining with 20 µL/100 mL ethidium bromide (10 mg/mL) and UV visualization by Gel Doc™ XR<sup>+</sup> imager (Bio-Rad, UK).

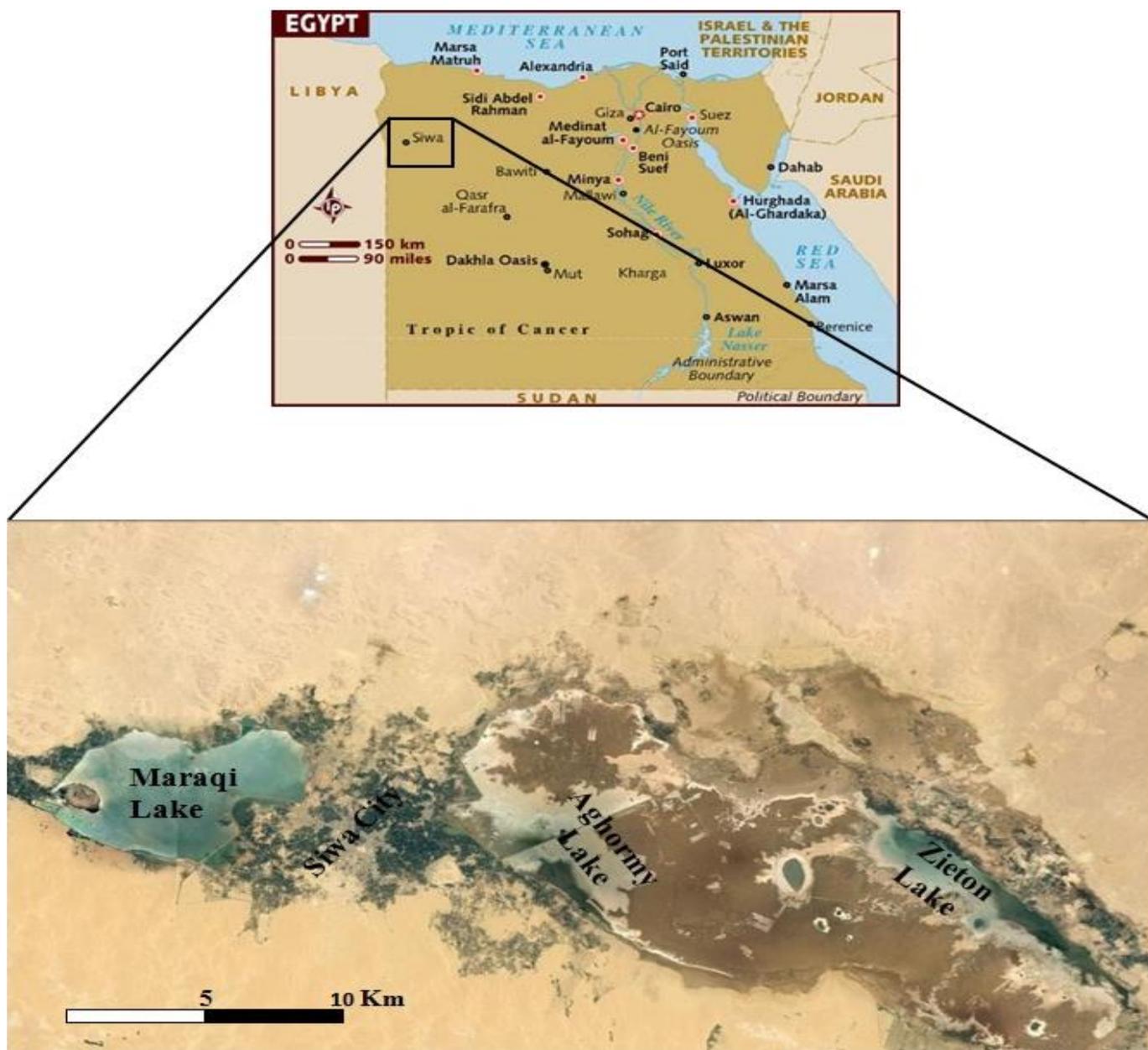
PCR amplifications of bacterial 16S rRNA gene were done, using the primers 341F-GC, 5'- CgC CCg CCg CgC CCC gCg CCC gTC CCg CCg CCC CCg CCC g CCT ACg ggA ggC AgC Ag -3' and 907R, 5'- CCg TCA ATT CMT TTg AgT TT -3' (Nubel et al., 1996). All primers were manufactured by nucleotide synthesizer and purified through HPLC (Life technology, England).

The PCR mixture contained 2.5 mM dNTP each, 10X Ex Taq™ buffer (Mg<sup>2+</sup> free), 25 mM MgCl<sub>2</sub>, 0.25 µM of each primer, 250 U Takara Ex-Taq™ Polymerase (Takara, Japan) and 500 ng DNA template. PCR was performed on a ProFlex™ PCR System (Life technology, USA), using an initial denaturation step of 3 min at 95°C. The touchdown PCR reaction continued with 30 cycles of 50 s at 95°C, with starting annealing temperature, 57°C, followed by decreasing 0.1°C every cycle, and 1 min at 72°C, with a final extension for 12 min at 72°C. Moreover, the bias in PCR was minimized by doing PCR replicates, for each lake water sample, using number of cycles ranged from 25 to 30 cycles (Suzuki and Giovannoni, 1996). These conditions gave the flexibility for the primers to anneal with various 16S rRNA gene templates with different GC contents and obtained PCR amplicons with sizes ranged from 547 to 561 bp. 16S rRNA gene amplicons were stained with ethidium bromide and visualized on 1.28 % agarose gel.

### Denatured gradient gel electrophoresis, DGGE

DGGE was done according to the method of Muyzer et al. (1993), with modifications. The 16S rRNA gene amplicons were run on 6% polyacrylamide gel, the ratio of acrylamide to bisacrylamide was 37.5:1; 40% w/v, in 7 L of 1X TAE running buffer (Tris-acetate, 0.04 M, EDTA, 0.002 M, pH 8.5).

Electrophoresis was run for 17 h at 120 V and 35 Amp under a constant temperature of 60°C in a DCode™ Universal Mutation Detection System (Bio-Rad, UK). After electrophoresis, the gel was



**Figure 1.** A map showing the sites of sampling at Siwa Lakes, Egypt.

stained with 20 µL/100 mL ethidium bromide concentration, 10 mg/mL, for 20 min, and visualized under UV light. The recovered clear bands were purified using the QIAquick® PCR purification kit (Catalog no. 28104, Qiagen, Germany) and analyzed by sequencing. Sequencing was done by the Applied Biosystems 3500 Genetic analyzer sequencer (Hitachi, Japan).

**DGGE analyses**

DGGE patterns were analyzed by binary matrix, using AlphaView Software v.3.4.0 (<http://www.proteinsimple.com/>). The designation

of the band-classes was based on their position in the gel patterns. The DGGE fingerprinting was manually scored by the presence (1) and absence (0) of co-migrating bands, independent of intensity. To ensure gel-to-gel comparability, the bands, common and specific, were furthermore inspected manually for consistency. Distances between gel patterns were determined using the cluster analysis with Euclidian measure. Similarity analysis of DGGE profiles was done using both of Jaccard coefficient,  $S_{Jaccard}$  and Dice coefficient,  $S_{Dice}$ , which were calculated according to the formulas:

$$S_{Jaccard} = N_{AB} / (N_A + N_B - N_{AB})$$

$$S_{Dice} = 2 N_{AB} / (N_A + N_B)$$

**Table 1.** Binary matrix of DGGE phylotypes and sequence accession numbers.

Phylotypes	Lakes			Accession numbers
	Aghormy	Zieton	Maraqı	
Siwa 1.Z.M	0	1	1	LC164659
Siwa 2.Z	0	1	0	LC164660
Siwa 3.Z	0	1	0	LC164661
Siwa 4.M	0	0	1	LC164662
Siwa 5.A	1	0	0	LC164663
Siwa 6.A	1	0	0	LC164664
Siwa 7.A	1	0	0	LC164665
Siwa 8.Z	0	1	0	LC164666
Siwa 9.A	1	0	0	LC164667
Siwa 10.Z	0	1	0	LC164668
Siwa 11.A	1	0	0	LC164669
Siwa 12.A.Z	1	1	0	LC164670

Where  $N_{AB}$  is the number of bands common to both patterns;  $N_A$  and  $N_B$ , representing the total number of bands in sample A and B, respectively.

Sequences were analyzed by FASTA screening to determine their similarity to the known sequences in the DNA database (<http://www.ebi.ac.uk/Tools/sss/fastal/>).

Phylogenetic tree was constructed through two bioinformatics processes. In the first process, the nucleotide sequences of the recovered rRNA gene phylotypes and their homologous sequences from the DNA database were aligned using the online program "Clustal Omega" software (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). In the second process, the aligned sequences, including the sequence gaps, were submitted to MEGA software, V. 6.0.6, (<http://www.megasoftware.net/>), for construction of consensus phylogenetic tree, using maximum likelihood algorithm. The branching patterns of the constructed phylogenetic tree were confirmed by reconstruction of the phylogenies using the two other algorithms, neighbor joining and maximum parsimony. Bootstrap method, provided as a phylogeny test, in the MEGA software, was performed using a number of 500 bootstrap replications.

#### Nomenclature of phylotypes and sequence accession numbers

Each phylotype was marked by a number, showing its position on the DGGE profile, followed by the first letter of the source lake, where Aghormy, A, Zeiton, Z, and Maraqı, M, for example, phylotype 7.A, means phylotype number 7, belonging to Lake Aghormy. The phylotype synonym 1.Z.M indicated the phylotype number 1, which occurred in both of Zeiton and Maraqı Lakes, and so on.

The current 16S rRNA gene sequences have been registered in the DNA database under accession numbers listed in (Table 1).

## RESULTS AND DISCUSSION

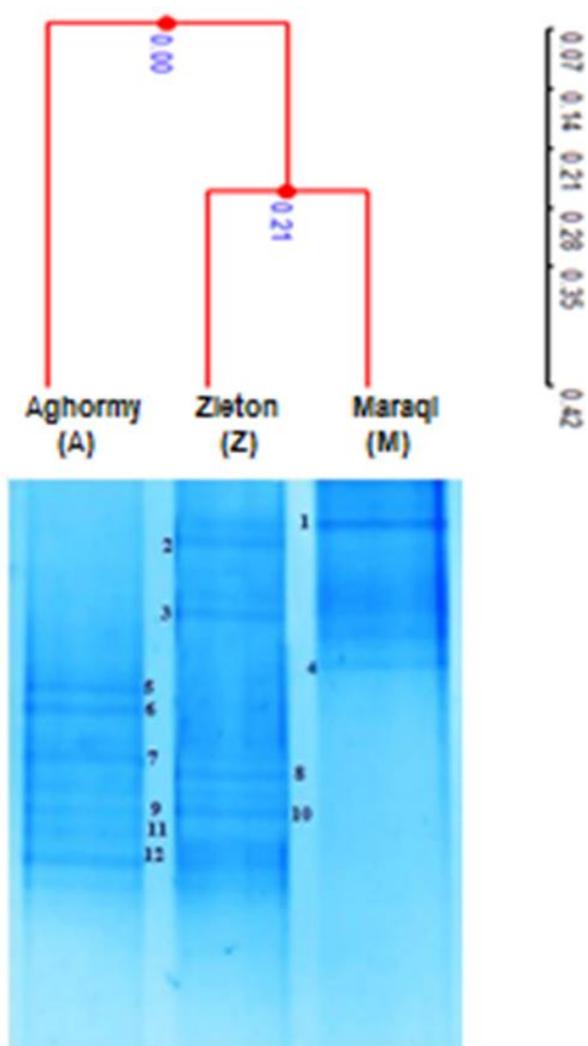
### DGGE displayed specific bacterial 16S rRNA gene phylotype patterns for the studied lakes

All genomic DNAs from the lakes showed positive PCR

amplifications of the bacterial 16S rRNA gene, variable regions, V3-V5. The DGGE displayed total of 12 bands, each band represented a phylotype. Each of the Lakes Aghormy and Zieton displayed 6 phylotypes (Figure 2 and Table 1). The Lake Maraqı had the lowest bacterial richness, representing only two phylotypes (Figure 2 and Table 1). Maraqı showed the highest accumulation of total dissolved solid contents, 280.45 gm/L, among the studied Siwa lakes, which may shaped the bacterial richness by occurrence of only limited species that could tolerate this very extreme environment (Abd El-Karim and Goher, 2016).

Some studies on hypersaline lakes showed numbers of DGGE bands greater than those of recorded in the current study. The DGGE banding patterns from hypersaline Lake Faro, Italy, showed a greatest difference of bands, 10 to 20 bands, was observed between depths of 0 and 30 m, respectively (Gugliandolo et al., 2011).

Richness of bacteria in Aran-Bidgol salt Lake, a thalassohaline lake in Iran showed 10 bands on DGGE profile (Makhdoumi-Kakhki et al., 2012). The results of 16S rRNA gene-based DGGE displayed 10 phylotypes from the hypersaline water of Lake Red, Romania (Borsodi et al., 2013). The PCR–DGGE method obtained 11 different bands, which were related to several distant taxonomic groups in central European hypersaline lakes (Keresztes et al., 2012). This difference in differential display of bacterial 16S rRNA gene DGGE patterns between current sampling lakes and other studied hypersaline lakes may be due to environmental conditions specific to each sampling location and methodology used in each study. Generally, the hypersaline environments have low bacterial diversity due to harsh conditions, which favor the dominance of specific bacterial community.



**Figure 2.** DGGE profiles of bacterial PCR-amplified 16S rRNA genes obtained from microbial mats collected from studied Siwa Lakes, Aghormy, A, Zieton, Z, and Maraql, M. Sequenced bands, phylotypes, are indicated by numbers, from 1 to 12, as follow: 1. Z.M; 2. Z; 3. Z; 4. M; 5. A; 6. A; 7. A; 8. Z; 9. A; 10. Z; 11. A; and 12. A.Z. Euclidian distances among DGGE profiles are shown.

### Dominance of bacterioidetes- and alphaproteobacteria-like phylotypes in the studied lakes

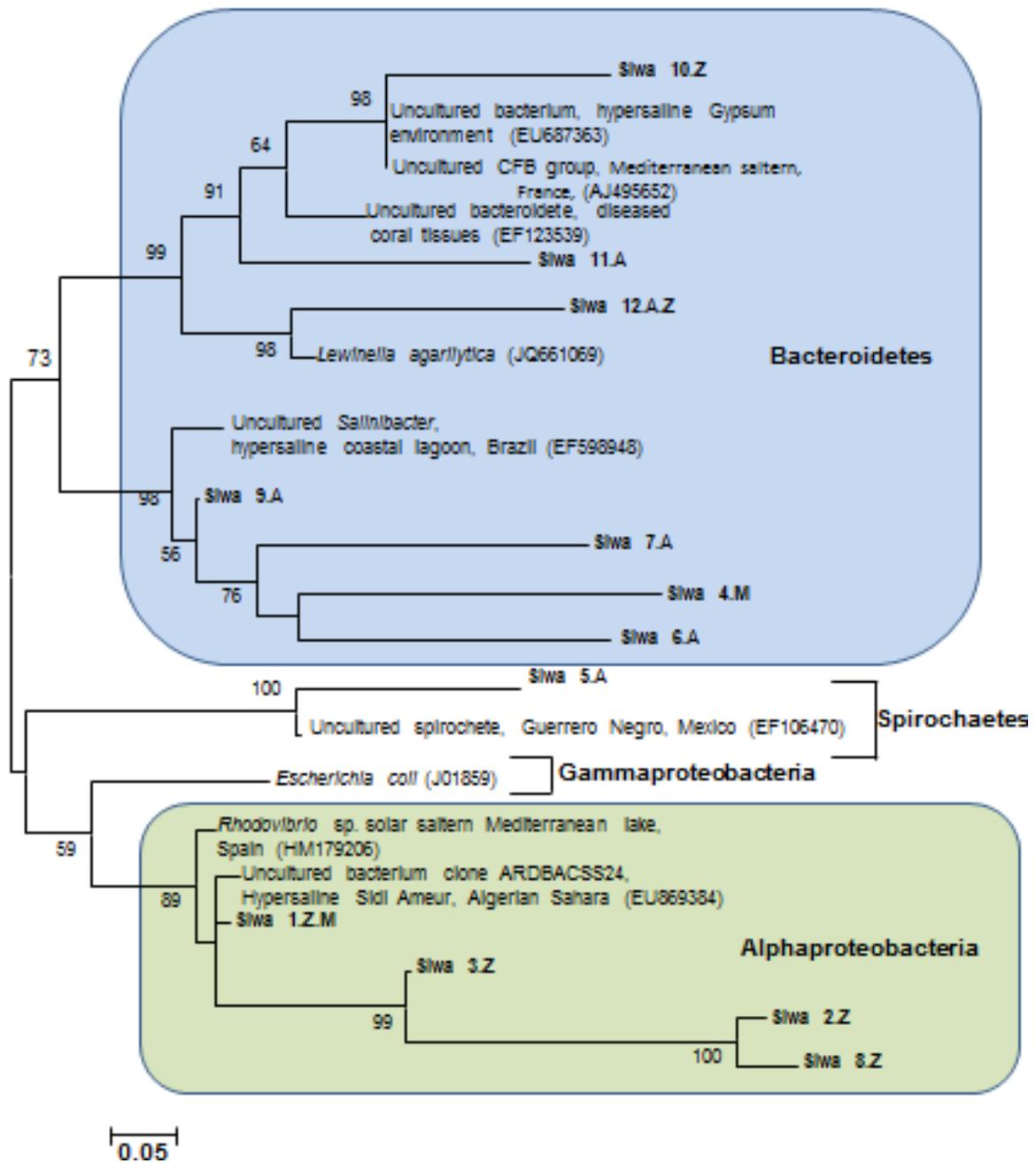
Phylogenetic analysis recorded 7 phylotypes, which were affiliated to the phylum Bacterioidetes (Figure 3). Bacterioidetes occur in wide range of hypersaline habitats (Oren, 2002; Gugliandolo et al., 2011; Borsodi et al., 2013). The phylotypes, 6.A, 7.A, 9.A and 4.M formed unique phylogenetic cluster with uncultured *Salinibacter* (Figure 3). Halobacterial genus, *Salinibacter*, has since been occurred ubiquitously in numerous hypersaline

environments (Anton et al., 2008; Makhdoumi-Kakhki et al., 2012). *Salinibacter* survives in these harsh environments because of its adaptations in order to cope with the high salt concentrations (Mongodin et al., 2005). It was found that *S. ruber* made up from 5 to 25% of the total prokaryotic community of the Spanish saltern ponds (Anton et al., 2002). The phylotype, 12.A.Z, formed monophyletic clade with *Lewinella agarilytica* (Figure 3) (Buerger et al., 2012). The genus *Lewinella* was proposed by Sly et al. (1998) to encompass the marine species of the genus *Herpetosiphon* (Holt and Lewin, 1968) under the phylum Bacterioidetes. The current phylotype, 12.A.Z represented the first record of *Lewinella*-like phylotypes in hypersaline inland lakes.

The phylotypes, 1.Z.M, 2.Z, 3.Z and 8.Z were located in the branch of Alphaproteobacteria and linked with *Rhodovibrio* sp. from a saltern Mediterranean lake and uncultured Alphaproteobacterium from the hypersaline Sidi Ameur, Algerian Sahara (Moune et al., 2003; Boutaiba et al., 2011). Alphaproteobacteria is a diverse taxon and comprises several phototrophic genera, which occur in hypersaline habitats (Edbeib et al., 2016). The genus *Rhodovibrio* represents 35.3% of the total bacterial composition in Aran-Bidgol salt, the largest hypersaline lake in Iran (Makhdoumi-Kakhki et al., 2012). On the other hand, the current phylotypes had homologies, 85 to 90%, with corresponding species in the branches of Bacterioidetes and Alphaproteobacteria, representing new phylogenetic lineages, and consequently, expanding the global diversity of these bacterial groups in hypersaline environments.

### Composition of 16S rRNA gene phylotypes implicates endemic bacterial communities within the studied lakes

The bacterial phylotype compositions were different among the studied lakes, showing very low similarity indices (Table 2). The similarity matrix value between Aghormy and Maraql was zero, representing completely different bacterial communities. These results may implicate that each lake has the special physicochemical characteristics, which may shape the bacterial composition, to be specific for each lake (Abd El-Karim and Goher, 2016). This concept was strengthened by co-occurrence of Alphaproteobacteria-like phylotype, 1.Z.M, in the Lakes Zieton and Maraql, while lacked in Lake Aghormy (Figure 3). Moreover, the recorded bacterial phylotypes showed distant phylogenetic lineages within the branches of Bacterioidetes and Alphaproteobacteria (Figure 3). On the other hand, the phylotype 5.A, characterized only the Aghormy Lake, forming a monophyletic clade with uncultured spirochaete isolated from hypersaline site, Guerrero Negro, Mexico (Figure 3) (Sahl et al., 2008). The spirochaetes have phylogenetic



**Figure 3.** A consensus phylogenetic tree based on 16S rRNA gene sequences of current recorded bacterial phylotypes, from the studied Siwa lakes; beside their corresponding sequences from database. Bootstrap values, more than 50%, of compared algorithms, are indicated at the branch roots. The bar represents 0.05 changes per nucleotide. Accession numbers of database extracted sequences are in brackets.

**Table 2.** Similarity matrix, using Jaccard coefficient (Dice coefficient).

Lakes	Aghormy	Zieton	Maraqı
Aghormy	100.0	-	-
Zieton	9.0 (16.6)	100.0	-
Maraqı	0(0)	14.28 (25)	100.0

relationships at the level of gross phenotypic characteristics. They possess some genomic features that help those bacteria in living at hypersaline habitats (DasSarma and DasSarma, 2012).

## Conclusion

The approach of uncultured-dependent PCR/DGGE followed by sequencing of DGGE bands, targeting the 16S rRNA gene, was applied to study bacterial richness in three Lakes, Aghormy, Zeiton and Maraqui, Siwa oasis, Egypt. The bacterial compositions in all three lakes had low diversity, a common characteristic of hypersaline environments. Bacterioidetes-like phylotypes dominated in all lakes, while Alphaproteobacteria-like phylotypes were occurred in Zeiton and Maraqui. Each lake showed novel endemic bacterial phylotype composition, with low nucleotide homologies with other previous studied halophilic taxons. However, this study opened a window to explore the bacterial phylotypes in hypersaline lakes of western desert, Egypt. Future phylogenetic studies must be done in order to cover the total diversity of all microbial communities, from other domains, archaea and eukaryotes, living within those lakes.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGMENTS

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