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DIVERSITY OF CULTIVABLE MICROORGANISMS IN THE EASTERN PART OF URMIA SALT LAKE, IRAN

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ABSTRACT

In this study we employed culture techniques to study microbial diversity in Urmia Lake, a hypersaline lake in northwest of Iran. Water, soil and salt samples were taken from the Eastern part of Urmia Salt Lake in September 2011. A total of 11 water samples and 30 soil and salt samples were taken from 41 sites in the Lake. Bacterial isolates were cultured on different growth media and taxonomically affiliated based on their 16S rDNA gene sequence. Three hundred bacterial isolates were obtained from samples collected. Of these, 53 bacterial isolates were selected for sequencing and phylogenetic analysis, based on their growth characteristics and colony morphology. Results showed that these 53 isolates represented 39 species, belonging to 18 genera (*Bacillus*, *Oceanobacillus*, *Thalassobacillus*, *Planomicrobium*, *Halobacillus*, *Planococcus*, *Terribacillus*, *Staphylococcus*, *Piscibacillus*, *Virgibacillus*, *Gracilibacillus*, *Ornithinibacillus*, *Halomonas*, *Pseudomonas*, *Providencia*, *Salicola*, *Psychrobacter*, *Kocuria*) and they were from 9 families (*Bacillaceae*, *Planococcaceae*, *Staphylococcaceae*, *Halomonadaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Moraxellaceae*, *Alteromonadaceae*, *Micrococcaceae*) pertaining to three phyla (*Actinobacteria* 1.8%, *Firmicutes* 78.6%, *Proteobacteria* 21.4%). The present study showed that Urmia Lake is a rich source for moderately halophilic and halotolerant bacteria. The phylogenetic analysis of sequences from Urmia Lake had some common 16S rDNA sequences from other hypersaline lakes previously reported.

Keywords: Halophilic bacteria, phylogenetic diversity, Urmia salt lake, 16S rDNA gene

INTRODUCTION

Saline habitats are globally distributed on Earth and are valuable sources of novel microorganisms **(Hongchen *et al.*, 2007)**. Hypersaline lakes, with salinities at or near saturation are biologically very productive ecosystems. They are extreme habitats in terms of NaCl concentration and yet maintain remarkably high cell densities ($\geq 10^7$ cells per mL) **(Burns *et al.*, 2004; Makhdoumi-Kakhki *et al.*, 2012)**. The salinity gradient in hypersaline environments is a common phenomenon, due to the evaporation of water. **(Houda *et al.*, 2010)**. The salinity gradients affect the structure and the composition of microbial communities allowing the growth of highly specialized organisms from all three domains of life.

In terms of salt requirements, microbes can be considered halotolerant or halophilic. Halotolerant microorganisms have no specific requirement for salt, other than usual 100-200 mM NaCl needed by all organisms. Organisms that require salt for growth are called halophiles, and they are classified into slight, moderate and extreme halophiles. The moderate halophiles have 0.2-0.5 M NaCl requirement and will grow in up to 3.5-4.0 M NaCl, extreme and slight halophiles grow in media containing 2.5–5.2 M salt and 0.2–0.5 M salt respectively **(Russell 1989).**

Besides, halophilic/halotolerant microorganisms have potential applications in various fields of biotechnology **(Dastgheib *et al.*, 2012; Delgado-García *et al.*, 2012; Llamas *et al.*, 2012; Rohban *et al.*, 2009; Tang *et al.*, 2011)** increasing the need for studies of the microbial diversity of hypersaline environments have encouraged. Identification of the organism‘s presence, assessment of their numeral importance, and cultivation of the dominant organisms in pure culture are the first important steps towards understanding the ecology of hypersaline lakes **(Burns *et al.*, 2004).**

The geographic region of Iran, is rich in hypersaline lakes and marshlands such as Howz-Soltan, Aran-Bidgol, Urmia, Maharloo Salt Lakes and Gomishan wetland whose microbial populations need to be elucidated. Urmia Salt Lake is located in the northwest of Iran. In many respects of morphology, sediments and chemistry, It resembles the Great Salt Lake in the western USA (**Kelt and Shahrabi 1986**). The predominance of the Na^+ and Cl^- ions illustrates the thalassohaline character of Urmia lake (**Sorgeloos *et al.*, 1997**). The lake is divided into north and south parts separated by a causeway (**Teimouri *et al.*, 1998**), which has a gap that allows limited exchange of water between the two arms (**Abazopoulos *et al.*, 2006**). This lake is an endorheic or terminal lake which means that water departs the lake only by evaporation. As is generally the case, this leads to a salt water body and in the case of Urmia Lake, the salinity isquite high. During the last years, the depth of the lake has dramatically decreased to 6 m for various reasons, further concentrating salts in the lake, raising salinity to more than 200-300 g/L or higher in many locations (**Farzin *et al.*, 2012**).

In the 2011’s, Iranian researchers successfully isolated halophilic bacteria from Urmia Lake and indicated that the isolated bacteria belonged to two major taxa *Proteobacteria* and *Firmicutes* **(Zununi Vahed *et al.*, 2011)**. Urmia Salt Lake has a wealth of microbial diversity. Therefore, this study expanded our knowledge to all parts of the eastern side of the lake. Also, we have obtained a greater number of strains. Here, our objective was to investigate the diversity of moderately halophilic and halotolerant bacteria in water, sediment, salt and soil samples in the eastern parts of the Urmia Lake.

MATERIAL AND METHODS

Description of the study site

Urmia Salt Lake (37° 32′ N, 045° 43′ E) in the northwest of Iran is the second lake in the world after the Great Salt Lake in the western USA in terms of NaCl concentration. The average salinity is about 220 to 300 g/L, depending on various

space and time conditions (**Farzin *et al*., 2012**). It is 140 km long, 55 km wide and at most 18 m deep in its extreme extended limit (Figure1).

Figure 1 Urmia Lake, in northwest of Iran at Azarbayjan region. The arrows in this figure point towards the sampling sites.

Physico chemical characteristics of water samples

Water (50 mL from each site), soil and salt (50g from each site) samples were collected aseptically from different zones in the east of Urmia Lake in September 2011and stored at 4 °C until studied for the laboratory analysis (always within 24 h). The temperature and pH were measured at each sampling point using portable instruments. Water samples were mixed and the total salt concentration was determined by titration according to the method of Mohr (**Doughty, 1924**). The contents of various ions were measured $Cl^-$by titration with $AgNO_3$ and Mg^{2+} by atomic absorption spectrophotometer, Na^+ by flame spectrophotometry and Ca^{2+} by complexometry method using EDTA (**Sheen and Kahler, 1938**).

Cultivation and identification of bacterial strains

A surface-spread plating method was used to determine the numbers of microorganisms in the samples. Enrichment and isolation of halotolerant and moderately halophilic bacteria were performed in two growth media. The soil and salt samples were serially diluted up to 10^{-6} and water samples were directly plated according to **Burns *et al*. (2004)**.
From the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions 100 µl of each, were surface-plated on moderate halophilic medium (MH)[a nutrient-rich and moderately saline (12%) medium for aerobic heterotrophic bacteria] containing (g/L); yeast extract (10), proteose peptone (5), glucose (1), NaCl (100), $MgCl_2.H_2O$ (7), $MgSO_4.7H_2O$ (1), $CaCl_2.2H_2O$ (0.36), KCl (2), $NaHCO_3$(0.06), NaBr (0.026) **(Caton *et al*., 2004; Vreeland, 2013)** and modified Sea water Nutrient Agar (SWN) (g/L); NaCl (20), $MgCl_2.6H_2O$ (3),$MgSO_4.7H_2O$ (5), $CaCl_2.7H_2O$ (0.05), KCl(0.5), peptone(5), yeast extract (1), meat extract (2) **(Bowman *et al*.,2003).**
The final pH of each medium was adjusted to 7.2 with 1M NaOH that corresponded with the characteristic pH value of the lake water. The plates were incubated at 34°C and counted for 3 weeks. Growth was scored according to the color, size and morphology of the colonies. In order to avoid sequencing of several identical bacterial isolates from the same media, only the colonies with different morphology were isolated after 3 weeks. We have selected and subcultured different colonies grown on media, and used them for further investigation.
Microbial cultures were stored at −80°C in the isolation medium (SWN and MH media) supplemented with 20% glycerol. Phenotypic characteristics of individual isolates including Gram-staining, cell morphology, catalase and oxidase activities were determined using standard methods **(Prescott *et al*., 2002)**. Salt tolerance of the isolates were assayed by plating each of them onto 5% yeast extract agar at pH 7.2 without NaCl. Isolates were screened for their salt tolerance efficacy level by plating each of the isolates onto 5% yeast extract agar at pH 7.2 supplemented with NaCl to total concentrations of 0.5; 3; 5;7.5; 10; 15; 20 and 25% (w/v). Cultures were incubated in aerobic conditions at 34°C for up to 3 days.

DNA extraction and PCR amplification of the 16S rDNA genes

Genomic DNA was extracted from log-phase cells according to Marmur (1961). The 16S rDNA gene was amplified by PCR using primers 1492R (5´-GGTTACCTTGTTACGACTT-3´) (**Turner *et al*., 1999**) and 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (**Lane *et al*., 1991**). The reaction mixture contained 0.5 µL (10mM) of each deoxynucleoside triphosphate, 1U of *Taq* DNA polymerase, 5 µL buffer (5X), 5µL $MgCl_2$ (20 mM), 1µL (10 pM) of each primer and about 20 ng of genomic DNA template in a total volume of 50 µL. PCR products were checked on a 1% agarose gel stained with ethidium bromide under UV excitation. The PCR conditions were as follows; 95°C for 5 min, followed by 30 cycles of 94°C for 60 s, 50°C for 60 s and 72°C for 60 s, and a final extension at 72°C for 7 min. The PCR products were purified with the GenElute™ PCR Clean-Up Kit (sigma aldrich).

Sequencing and sequence analysis

The purified PCR products were sequenced directionally by ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). Sequencing was performed using Sanger's method.
The sequences were identified by a similarity-based search using the EzTaxon-e web server (**http://eztaxon-e.ezbiocloud.net/; Kim *et al*., 2012**) and aligned using Clustal X2 **(Larkin *et al*., 2007).** To study the phylogenetic relationship among the strains and other species, weapplied neighbour-joining (NJ) method with Kimura two-state parameter and pairwise-deletion model using the MEGA5 software **(Tamura *et al*., 2011)**. Confidence levels for the phylogenetic trees were assessed by bootstrapping with 1000 replicates.

Nucleotide sequence accession numbers

The 16S rDNA gene sequences retrieved in this study have been deposited at Genbank (www.ncbi.nlm.nih.gov), and have accession numbers KF744338-KF744389 and KF770242-KF770247.
These strains were delivered to Iranian Biological Resource Center for deposition so that they are available for further studies.

RESULTS

Physicochemical analysis

The pH of all samples were neutral and they did not exhibit spatial or temporal variations. The study on these areas indicated that samples had a pH range between 7.2-7.5. The temperature of the sampling sites were between 20-25 °C, the salinity of the samples ranged from 23-32% total salts. The chemical analysis of the salt water samples were similar. Results are summarized in Table 1. Due to this similarity the water samples are mixed and used for further investigations.

Table 1 Chemical properties of water samples from Urmia Lake. Data presented are the mean of the concentrations of all the samples ± SD

Ion concentration (g. L^{-1})	Chemical Species
1.425±0.02	Fe^{2+}
80.654±0.04	Na^+
221.400±0.06	Cl^-
1.800±0.02	K^+
15.467±0.02	Mg^{2+}
0.800±0.03	Ca^{2+}
0.190±0.03	HCO_3^-
0.422±0.04	SO_4^{2-}
0.018±0.003	Ba^{2+}

Isolation and characterization of moderately halophilic and halotolerant bacteria

From the soil samples we obtained 5×10^6±0.4 CFU/g and 6×10^4±0.5 CFU/mL in the water samples. After several dilutions and repeated subculturing, 320 pure bacterial cultures isolated including 47.4%, 43.3% and 9.3% from water, soil and salt samples, respectively. They were obtained under aerobic conditions on the two growth media used. Morphological variation was observed when colonies were compared including smooth, circular, low-convex, transparent or translucent and different colors like red, orange-red, pale-pink, yellowish, cream or white. Approximately half of the isolates (51.8%) reported here were obtained with SWN medium (3% saline) and other strains were cultured on HM medium (12% salinity). The majority of the total isolates (77.4%) were Gram-positive, whereas the Gram negative's constituted only 22.6% of the total.
The phenotypic analysis of the isolates demonstrated that the culturable fraction of the microbial community was largely dominated by Gram-positive bacteria in all the samples studied. The ratio of bacilli to cocci was 6:1 in the Gram-positive bacteria.
The majority of the isolates were catalase positive (64.7%) and oxidase positive (66.3%). Characteristics of the selected isolates are summerized in Table 2. Most of the isolates (55.6%) were able to grow in the medium without salt thus indicating the halotolerant characteristic of these isolates while the rest required total salt of 0.2-0.5 M for their growth and were classified as moderate halophiles. Previous studies reported that Gram- positive bacteria especially the genus *Bacillus* were extensively presented in saline habitats **(Tang *et al.*, 2011; Berrada *et al*., 2012)**. Most of them were classified as halotolerant microorganisms which is in agreement with our results.

Table 2 Characteristics of the selected strains

	Strains	Number of strains	Strain No.	Source of strains	Medium type	Catalase	Oxidase	Cell and colony morphology
Gram positive	*Bacillus safensis*	8	G7	water	SW	+	+	Cream, small, rod-coccus-shaped
			B5	water	SW	-	+	Cream, small, rod-shaped
			C3	water	SW	+	+	Translucent white, large, rod-shaped
			F2	water	sw	-	-	Cream, medium, rod-shaped
			GB2	salt	sw	+	+	Smooth, pink-cream, rod-shaped
			K4	water	SW	+	+	Light-orange-red, circular, medium, rod-shaped
			SB1	soil	SW	+	-	Pale white, medium, circular,rod-shaped
			I6	water	SW	-	+	Snowy white, large, rod-shaped
	Oceanobacillus picturae	3	H10	water	HM	+	+	Pale yellow, medium, rod-shaped
			M47	water	HM	+	+	Lemon yellow, medium, rod-shaped
			E4	water	HM	+	+	White, medium, rod-shaped
	Thalassobacillus devorans	1	H7	water	HM	+	+	Lemon yellow, low-convex, medium, rod-shaped
	Bacillus aerophilus	1	I1	water	SW	+	+	Snowy white, medium, rod-shaped
	Bacillus atrophaeus	1	RCI	soil	SW	+	-	Pale white, medium, rod-shaped
	Bacillus pumilus	2	K5	water	HM	+	+	Cream, small, low-convex, rod-shaped
			M9	water	SW	-	+	Smooth and pink-cream, small, rod-shaped
	Halobacillus salsuginis	1	GA4	soil	HM	+	-	Orange-red, medium, rod-shaped
	Halobacillus litoralis	1	M42	water	HM	+	-	Lemon yellow, medium, rod-shaped
	Bacillus hwajinpoensis	3	GD4	soil	SW	+	+	Light pink, large, rod-shaped
			B7	water	HM	-	+	Cream, medium, rod-shaped
			M2	water	SW	-	-	Yellow, small, rod-shaped
	Bacillus aryabhattai	1	GA5	soil	SW	+	+	Pale red, large, rod-shaped
	Planococcus maritmus	1	KD4	soil	SW	-	-	Orange-red, large, coccoid-shaped
	Bacillus jeotgali	1	M36	water	SW	+	+	Orange-red, medium, rod-shaped
	Terribacillus aidingensis	1	MB5	soil	HM	+	-	Cream, medium, rod-shaped
	Staphylococcus hominis	1	MD1	salt	HM	+	-	Snowy white, large, rod-shaped
	Planococcus salinarum	1	RA4	soil	SW	+	+	Smooth and pink-cream,medium, rod-shaped
	Planococcus rifietoensis	1	F4	water	HM	+	-	Cream, medium, rod-shaped
	Pontibacillus hungwhensis	1	N7	water	HM	+	-	Pale yellow, small, rod-shaped
	Piscibacillus halophilus	1	B16	water	HM	+	+	Cream, medium, rod-shaped
	Virgibacillus byunsanensis	1	KB1	soil	HM	+	+	Snowy white, large, rod-shaped
	Gracilibacillus dipsosauri	1	N13	water	HM	-	-	Light yellow, small, rod-shaped
	Virgibacillus necropolis	1	SE2	salt	HM	+	+	Translucent white, large, rod-shaped
	Ornithinibacillus scapharcae	1	BD5	soil	SW	+	+	Cream, medium, rod-shaped
	Bacillus thuringiensis	1	M8	water	SW	-	-	Light white, medium, rod-shaped
	Bacillus horikoshii	1	B1	water	SW	-	-	Cream, medium, rod-shaped
	Bacillus sonorensis	1	M16	water	HM	+	-	Orange-red, small, rod-shaped
	Bacillus vietnamensis	1	M29	water	SW	-	-	Light –pink, small, rod-shaped
	Bacillus thioparans	1	RF2	salt	SW	-	-	Pale yellow, medium, rod-shaped
	Kocuria rosea	1	M31	water	SW	+	+	Orange-red, medium, Coccoid-shaped
Gram negative	*Planomicrobium okeanokoites*	2	G2	water	SW	+	+	Light –pink, medium, coccoid-shaped
			KA6	soil	HM	+	+	Orange-red, large, rod-shaped
	Halomonas ventosae	1	RF6	salt	HM	-	+	Cream, medium, rod-shaped
	Pseudomonas xanthomarina	1	SD1	soil	SW	-	+	Translucent white, large, rod-shaped
	Halomonas sulfidaeris	1	MB10	soil	SW	+	+	Light cream, medium, rod-shaped
	Providencia vermicola	1	B6	water	SW	+	-	Cream,large, rod-shaped
	Psychrobacter faecalis	1	RF1	salt	HM	+	-	Cream, medium, rod-coccus
	Salicola salis	5	M29	water	SW	-	-	Orange-red, medium, rod-shaped
			B10	water	HM	-	-	Cream, small, rod-shaped
			F14	water	HM	+	+	Light yellow, small, rod-shaped
			N3	water	HM	-	+	Transparent Cream, small, low-convex, rod-shaped
			F15	water	HM	+	+	Light yellow, medium, rod-shaped

Identification of the strains

Among the total 320 isolates, 53 isolates produced morphologically different colonies and were selected for taxonomic identification based on 16S rDNA gene sequences. The partial Sequence of 16S rDNA gene was obtained and analysed (Table 2). These results and phenotypic properties of the isolated strains indicated that 53 moderately halophilic and halotolerant bacterial species were presented in the samples from Urmia Salt Lake.

Table 3 16S rDNA sequencing analysis of the starins

Accession number	Tentative identification based on nearest neighbor by the EzTaxon-e web server	Similarity with nearest type strain(%)[a]	length (bp) of sequenced	Strain
KF744338	*Bacillus safensis* FO-036b^T	99.7	1045	G7 strain IBRC-M
KF770242	*Oceanobacillus picturae* LMG 19492^T	99.0	884	H10 strain IBRC-M
KF744340	*Thalassobacillus devorans* G-19.1^T	98.6	951	H7 strain IBRC-M
KF744341	*Bacillus aerophilus* 2BK^T	100.0	869	I1 strain IBRC-M
KF744342	*Bacillus pumilus* ATCC 7061^T	99.8	915	K5 strain IBRC-M
KF744343	*Bacillus pumilus* ATCC 7061^T	99.9	869	M9 strain IBRC-M
KF744344	*Planomicrobium okeanokoites* IFO 12536^T	98.7	610	G2 strain IBRC-M
KF744345	*Halobacillus salsuginis* JSM 078133^T	98.6	730	GA4 strain IBRC-M
KF744346	*Bacillus hwajinpoensis* SW-72^T	98.9	710	GD4 strain IBRC-M
KF744347	*Bacillus aryabhattai* B8W22^T	100	670	GA5 strain IBRC-M
KF744348	*Planococcus maritmus* TF-9^T	99.1	680	KD4 strain IBRC-M
KF744349	*Bacillus jeotgali* YKJ-10^T	100.0	663	M36 strain IBRC-M
KF744350	*Terribacillus aidingensis* YI7-61^T	99.5	660	MB5 strain IBRC-M
KF744351	*Staphylococcus hominis* subsp. *hominis* DSM 20328^T	99.7	700	MD1 strain IBRC-M
KF744352	*Planococcus salinarum* ISL-16^T	99.7	690	RA4 strain IBRC-M
KF744353	*Bacillus atrophaeus* 9070^T	99.8	654	RC1 strain IBRC-M
KF744354	*Pseudomonas xanthomarina KMM 1447T*	98.7	700	SD1 strain IBRC-M
KF744355	*Planococcus rifietoensis* M8^T	99.9	924	F4 strain IBRC-M
KF744356	*Bacillus safensis*FO-036b^T	99.8	937	B5 strain IBRC-M
KF744357	*Providencia vermicola* OP1^T	99.2	798	B6 strain IBRC-M
KF744358	*Bacillus safensis FO*-036b^T	99.6	790	C3 strain IBRC-M
KF744359	*Oceanobacillus picturae* LMG 19492^T	99.4	758	E4 strain IBRC-M
KF744360	*Bacillus hwajinpoensis* SW-72^T	99.5	791	M2 strain IBRC-M
KF744361	*Piscibacillus halophilus* HS224^T	100	792	B16 strain IBRC-M
KF744362	*Pontibacillus hungwhensis* BH030062^T	99.9	761	N7 strain IBRC-M
KF744363	*Halomonas sulfidaeris* ATCC BAA-803^T	99.5	777	MB10 strain IBRC-M
KF744364	*Halobacillus litoralis* SL-4^T	99.9	771	M42 strain IBRC-M
KF744365	*Planomicrobium okeanokoites* IFO 12536^T	98.7	766	KA6 strain IBRC-M
KF770245	*Bacillus hwajinpoensis* SW-72^T	99.2	762	B7 strain IBRC-M
KF744366	*Salicola salis* B2^T	99.9	795	F14 strain IBRC-M
KF744367	*Salicola salis* B2^T	99.9	842	F15 strain IBRC-M
KF744368	*Pontibacillus hungwhensis* BH030062^T	99.9	850	F7 strain IBRC-M
KF744369	*Salicola salis* B2^T	99.8	829	M59 strain IBRC-M
KF744370	*Salicola salis* B2^T	99.9	825	B10 strain IBRC-M
KF744371	*Gracilibacillus dipsosauri* DD1^T	99.7	766	N13 strain IBRC-M
KF744372	*Virgibacillus necropolis* LMG 19488^T	98.4	1409	SE2 strain IBRC-M
KF744373	*Ornithinibacillus scapharcae* TW25^T	100.0	763	BD5 strain IBRC-M
KF744374	*Kocuria rosea* DSM 20447^T	99.6	750	M31 strain IBRC-M
KF744375	*Bacillus thuringiensis* ATCC 10792^T	100.0	816	M8 strain IBRC-M
KF744376	*Psychrobacter faecalis* Iso-46^T	99.6	809	RF1 strain IBRC-M
KF744389	*Bacillus safensis* FO-036b^T	99.7	750	SB1 strain IBRC-M
KF744377	*Halomonas ventosae* Al12^T	98.8	791	RF6 strain IBRC-M
KF744378	*Bacillus safensis* FO-036b^T	99.7	750	K4 strain IBRC-M
KF744379	*Bacillus horikoshii* DSM 8719^T	98.8	751	B1 strain IBRC-M
KF744380	*Bacillus safensis* FO-036b^T	100.0	750	F2 strain IBRC-M
KF744381	*Bacillus safensis* FO-036b^T	100.0	763	GB2 strain IBRC-M
KF770246	*Bacillus safensis* FO-036b^T	99.0	741	I6 strain IBRC-M
KF744382	*Bacillus sonorensis* NRRLB-23154^T	98.1	750	M16 strain IBRC-M
KF744384	*Bacillus vietnamensis* 15-1^T	97.3	750	M29 strain IBRC-M
KF744385	*Salicola salis* B2^T	99.6	774	N3 strain IBRC-M
KF744386	*Bacillus thioparans* BMP-1^T	99.7	750	RF2 strain IBRC-M
KF744387	*Oceanobacillus picturae* LMG 19492^T	98.5	756	M47 strain IBRC-M
KF770247	*Virgibacillus byunsanensis* ISL-24^T	97.5	1452	KB1 strain IBRC-M

[a]On the basis of pairwise comparison of the 16S rDNA gene sequences by the EzTaxon-e web server

In this study, using 16S rDNA sequences as the main tool for identifcation of moderately halophilic and halotolerant bacteria demonstrates that this method is helpful since 53 strains (17.0%) were identified at the species level with ≤ 100% similarity with their relative type strains.

The 16S rDNA gene sequences affiliated isolates 39 species of eighteen genera in the domain Bacteria. Bacterial isolates related to *Bacillus, Oceanobacillus, Thalassobacillus, Planomicrobium, Halobacillus, Planococcus, Terribacillus, Staphylococcus, Piscibacillus, Virgibacillus, Gracilibacillus, Ornithinibacillus, Halomonas, Pseudomonas, Providencia, Salicola, Psychrobacter, Kocuria* and nine families (*Bacillaceae, Planococcaceae, Staphylococcaceae, Halomonadaceae, Pseudomonadaceae, Enterobacteriaceae, Moraxellaceae, Alteromonadaceae, Micrococcaceae*) with the maximum identity between 97.3-100% as indicated in the Table 3. The phylogenetic placement of these sequences is presented in Figures 2, 3 and 4. As seen in the phylogenetic trees, by depicting the bootstrap values the results indicated that 53 strains belonged to three major groups, *Actinobacteria* 1.8%, *Firmicutes* 78.6% and *Proteobacteria* 21.4%.

Figure 2 Phylogenetic relationship of Urmia Lake isolates based on 16S rDNA gene homology from clustering with the *Firmicute.* The tree was constructed using the neighbor-joining method with Kimura 2-state parameter and pairwise-deletion model analyses implemented in the program MEGA version 5.1. The resultand tree topologies were evaluated by bootstrap analysis based on 1000 replicates. Numbers at nodes represent percentage levels of bootstrap support (%). The sequence of the type strain of *Lactobacillus* sp. FS60-1 (AB023836) was used as outgroup.

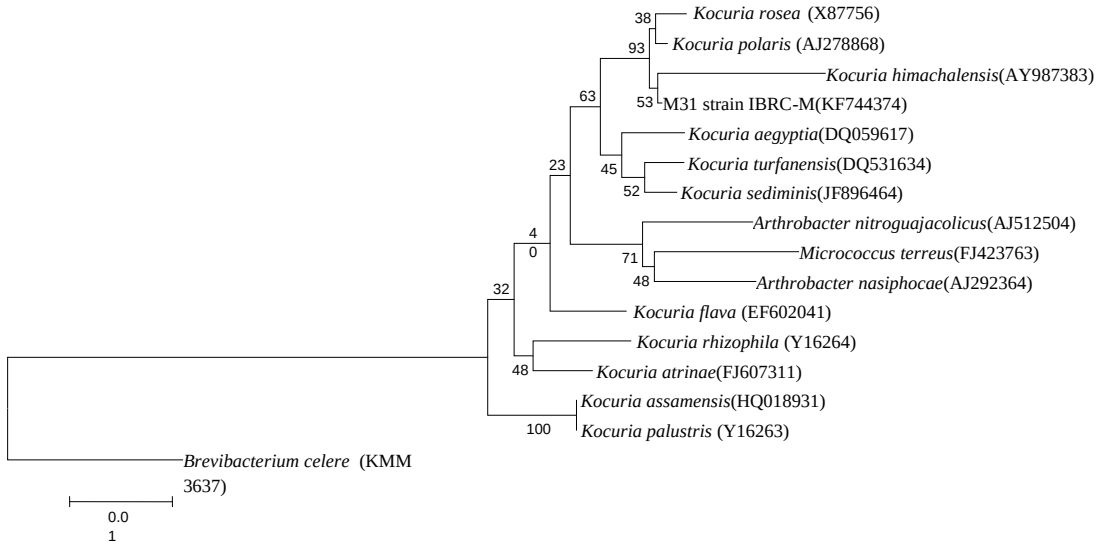

Figure3 Phylogenetic tree of the *Actinobacteria* inferred from a fragment of the 16S rDNA gene. The tree was inferred from a 16S rDNA gene sequence using Neighbor-joining method with Kimura 2-state parameter and pairwise-deletion model analyses implemented in the program MEGA version 5.1. Support for nodes in the tree corresponds to bootstrap values for 1000 pseudoreplicates. The tree has been arbitrarily rooted on the *Brevibacterium celere* strain KMM 3637

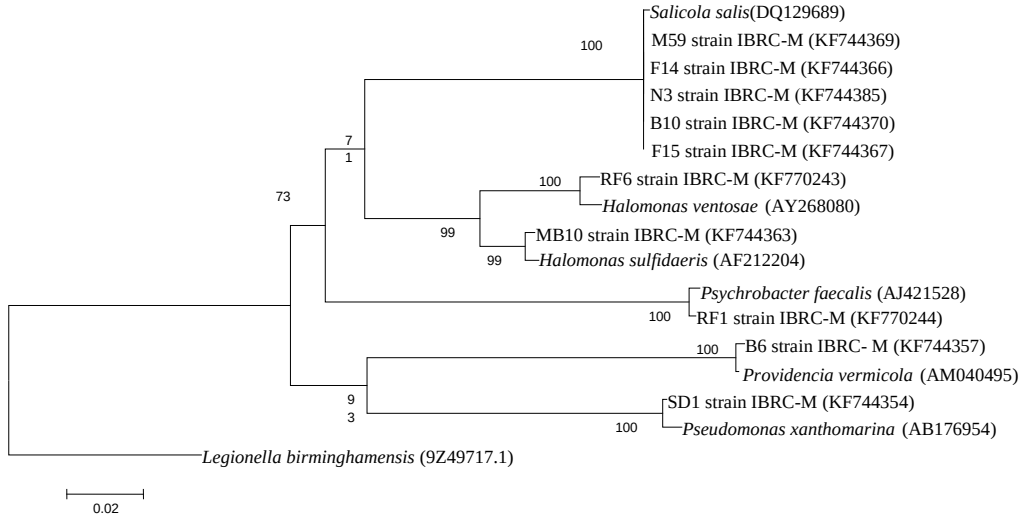

Figure4 Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of representative isolates affiliated with *Proteobacteria* with Kimura two parameter and pairwise-deletion model analyses implemented in the program MEGA version 5.1. The tree has been arbitrarily rooted on the *Legionella birminghamensis* 9Z49717.1

The single isolate (*Kocuria rosea*) was closely related to the phylum *Actinobacteria* belonging to the high G+C group of Gram-positive.

In the present study, *Firmicutes* belonging to the low G+C group were more diverse and abundant than Gram-negative *Proteobacteria* which is probably because of the inability of culturing Gram-negative bacteria from the lake. Our results showed that among the genera obtained from saline, soil and water samples, the dominant genus is *Bacillus* with 32 strains representing 20 species indicating that *Bacillus* is well adapted to saline environments.

The data indicate that culturable bacteria observed on SWN and HM media plates were mainly Gram-positive organisms related to *Bacillus, Thalassobacillus, Planococcus,Virgibacillus, Ornithinibacillus* and Gram-negative bacteria related to *Halomonas, Pseudomonas, Providencia, Salicola, Psychrobacter, Terribacillus, Staphylococcus, Oceanobacillus, Actinobacteria, Planomicrobium.*

DISCUSSION

On the basis of the chemical analysis of the water, saline water can be classified into two categories thalassohaline water (derived from seawater, with Na^+ and Cl^- as the predominant ions) and athalassohaline (with an ionic composition markedly influenced by the area where the pond developed (**Grant, 2004**). Athalassohaline waters usually contain higher concentrations of bivalent ions such as calcium and magnesium, in contrast to the relative dominance of monovalent ions (sodium and chloride) in sea-water (**Zafrilla *et al*., 2010**). Iran has a great diversity of hypersaline environments whose microbial population needs to be elucidated. Urmia salt lake is the largest hypersaline lake in Iran.

The results obtained from chemical analysis of saline water samples from Urmia Lake, which clearly show that Na^+ and Cl^- are the dominant ions, indicate that this lake is a thalassohaline environment. Furthermore, the present study shows that Urmia Lake is a rich source of moderately halophilic bacteria and halotolerant bacteria.

The microbial populations of many hypersaline environments have already been studied in different geographical regions. The most important ecosystems studied so far include the Great Salt Lake, Utah, USA (27-30% salinity), Sehline Sebkha Salt Lake in Tunisia (15–26%), the Dead Sea, the extremely alkaline brines of the Wadi Natrun, Egypt and lake Magadi, Kenya, Tunisian multi pond solar saltern with 5–15% of salt, Aran-Bidgol Lake (Iran), salt marshes, the solar salterns in Egypt, El Djerid Salt Lake (Tunisia) with NaCl concentration ranging from 15-26%, Lake Chaka in china (32.5% salinity), Howz Soltan Lake (Iran**) (Baati *et al*., 2010; Ghozlan *et al*., 2006; Jiang *et al*.,2006; Hedi *et al*., 2009; Hedi *et al*., 2014; 2006; Makhdoumi-Kakhki *et al*., 2012; Rohban *et al*., 2009; Weimer *et al*., 2009).**

Some of the isolates obtained from these hypersaline environments were common to Urmia Lake. Genus *Halomonas* were reported in all previously mentioned hypersaline environemnts. It is now becoming clear that *Halomonas* populations contribute to the prokaryotic communities at the highest salt concentrations and *Halomonas* have been adapted to hypersaline habitats. In contrast, *Planomicrobium, Terribacillus, Staphylococcus, Ornithinibacillus, Providencia, Psychrobacter* and *Kocuria* genera were unique to this study. Different results obtained in our study is suggested to be due to differences in salt concentration

and geographic conditions. These results indicate that the salinity gradients affect the structure and the composition of microbial communities.

According to halophilic strains reported by **Rohban *et al.*, (2009)**, members of the genera *Salicola, Halovibrio, Halomonas, Oceanobacillus, Thalassobacillus, Halobacillus, Virgibacillus, Gracilibacillus, Salinicoccus* and *Piscibacillus* were identfied from Howz-Soltan Lake, Iran. The phylogenetic analysis of bacteria from the Urmia Salt Lake was in accord with those reported by **Rohban *et al.*, (2009)** for Howz-Soltan Lake, whereas, genera *Halovibrio* and *Salinicoccus* were not present in our study.

Hedi *et al*., 2009 indicated that halophilic microorganisms isolated from El-Djerid Salt Lake containing up to 25% NaCl belonged to genera *Salicola, Pontibacillus, Halomonas, Marinococcus* and *Halobacillus*. The genera *Halomonas, Pontibacillus* and *Halobacillus* were common to our study.

Hedi *et al*., 2014 reported 77 bacterial strains and two archaeal strains from Sehline Sebkha Salt Lake (15–26% salinity) in Tunisia.

These bacterial strains related to *Halobacillus, Marinococcus, Pontibacillus, Bacillus, Salicola, Yeomjeonicoccus, Halomonas, Gracibacillus, Halovibrio, Pseudomonas* and Archaeal strains were identified as *Haloferax* and *Natrinema*. Of these bacteria, representatives of the genera *Halobacillus, Bacillus, Salicola, Halomonas, Gracibacillus, Pseudomonas* were common to both studies.

In **2011, Zununi Vahed *et al***., studied this lake but his samples were obtained from different wharfs of Saray Coast of Urmia Lake in December 2006 and July 2009. Due to water level variations of this lake and samples taken, there is a different between our samples and theirs in terms of salt constituent‘s concentrations and microorganisms inhabted there. They have isolated thirty seven strains from water and soil of Urmia Lake, thirty four of them stained Gram negative and three were Gram positive. These strains are affiliated to two major taxa *Gammaproteobacteria* including *Salicola, Pseudomonas, Marinobacter, Idiomarina, Halomonas* and *Firmicutes* including *Bacillus* and *Halobacillus*. They have used different media including MH medium, Halomonas medium, Marine agar (Difco), MGM medium with 2, 7, 15, 25% total salt concentrations, Luria Bertani (LB) with 7.5% NaCl and Nutrient agar with 10% NaCl (**Zununi Vahed *et al*., 2011**).

Here, we had taken water, soil and salt samples from 41 sites in the east of Urmia Salt Lake in 2011. Three hundred and twenty bacterial isolates were obtained in pure culture from these samples. Among 320 total isolates, 53 isolates were selected for taxonomic identification based on 16S rDNA gene sequences. The isolated strains are related to *Bacillus, Thalassobacillus, Planococcus,Virgibacillus, Ornithinibacillus, Halomonas ,Pseudomonas, Providencia, Salicola, Psychrobacter, Terribacillus, Staphylococcus, Oceanobacillus* and *Planomicrobium*. 16S rDNA sequence analyses showed that *Pseudomonas, Halomonas, Bacillus* and *Halobacillus* are common between the study by **Zununi Vahed *et al*., (2011)** from Urmia Lake and ours. Several isolates have only moderate 16S rDNA sequence similarity (97.3 to 98.9%) to their GenBank best match sequences from taxonomically well determined bacterial species, indicating their potential to be new species.

Since variations in salinity are an important difference between the lake waters. Due to drought and increased demands for agricultural water in the lake's basin, the salinity of the lake has raised during recent years. It is worth noting that salinity fluctuates substantially at the points where rivers discharge into the lake. Sudden changes in water salinity due to high precipitation at the very shallow shores of the lake are common and should be taken into account. Therefore, even in the saline highly Lake Urmia there are microenvironments where salinity differs substantially from the main body of the lake. These conditions could arguably provide the environmental stimuli leading toecological specialization of strains and, therefore, niche separation. This study indicate that Urmia Lake should harbor a prokaryotic diversity higher than and different from the current known diversity (**Zununi Vahed *et al*., 2011**). One reason is that the lake is subjected to high salinity which is caused by its dryness.

Recent studies on hypersaline environments including both the molecular and microbiological studies have revealed the presence of extremely halophilic microorganisms especially bacteria in a wide range of these saline environments such as salterns and hypersalin lakes and playa **(Boujelben *et al*., 2012; Hedi *et al*., 2009; Makhdoumi-Kakhki *et al*., 2012; Tang *et al*., 2011**).

Microbial diversity is difficult to measure in extreme environments due to the inability to culture many of the species, especially from hypersaline environments. It is perhaps not surprising that culture-independent approaches, such as oligonucleotide microarrays and sequencing 16S rDNA genes from denaturing gradient gel electrophoresis (DGGE) and clone libraries have identified far greater bacterial diversity than has been achieved using cultivation-based methods because many of the bacteria inhabiting saline environments cannot be easily cultivated to give a more comprehensive assessment of microbial diversity in these environments (**Lefebvre *et al*., 2006; Perreault *et al*., 2007; Tsiamis *et al*., 2008**). However, culture-independent approaches have the disadvantage that bacterial isolates are not obtained for further investigations and applications. There is an urgent need for new media and approaches for culturing halophilic and halotolerant bacteria from hypersaline environments (**Tang *et al.*, 2011**). A comparison of our findings and other culture-dependant studies suggests that the *Firmicutes* and *Proteobacteria* are indeed predominant among members of the cultivable bacterial community using a nutrient-rich and moderately saline medium for aerobic heterotrophic bacteria in a wide variety of hypersaline habitats worldwide (**Berrada *et al*., 2012; Hedi *et al*., 2009; Tang *et al*., 2011**). Therefore, Development of new media and growth conditions in the future will likely be resulted in the isolation of novel organisms from a unique hypersaline environment like Urmia salt Lake.

The results indicate that Urmia Lake is an important region for further investigation by combining different cultures conditions (media type and growth conditons) and other molecular methods.

CONCLUSION

In our study, Bacterial strains were isolated from the samples of Urmia Lake by using the conventional culture-dependent methods and investigated by using phylogenetic analysis based on 16S rDNA gene sequences. Results showed that strains represented 39 species belonging to 18 genera of 9 families. Although, the results of Urmia Lake were in overlaps with other lakes with similar habitats. But 16S rDNA gene similarity levels along with phenotypic characteristics suggest that some of the isolated strains could after more detailed analyses become representatives of novel species. The results suggested that three of the isolates (KB1, M16, M29) can be considered as new species, which means they should be characterized further using other methods.

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