

Conservation and Biodiversity Analysis of the Microalga *Dunaliella* in Shrinking Highly Saline Urmia Lake Based on Intron-sizing Method

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ABSTRACT

As the world's second saltiest lake, Urmia Lake is the main source of halotolerant unicellular microalga, *Dunaliella*, in Iran. Recently, this lake and, consequently, its biodiversity are being threatened environmentally. Hence collecting, preserving, and identification of indigenous microorganisms of the lake are of great importance. The objective of the present study was the molecular screening of *Dunaliella* isolates in Urmia Lake. For this purpose, 32 samples were taken from different geographical regions of the lake. Then, their molecular pattern was examined based on *18S rDNA* gene and intron-sizing method. Results based on conserved and species-specific primers of *18S rDNA* illustrated that, depending on the various parts of the lake, the genetic variation of *Dunaliella* population differs. The amplified pattern for individual isolates was similar to that previously described for *D. tertiolecta*, *D. bardawil* and *Dunaliella* sp. ARIINW-M1/2. Also, *18S rDNA* sequencing and phylogenetic analysis of five index isolates showed that the isolates *Dunaliella* sp. ABRIINW-Ch5, -Sh6.3 and -U1/1 were grouped with different intron lacking species of *Dunaliella*, ABRIINW-Ch3.1 was clustered with *Dunaliella* sp. ABRIINW-M1/2, while the isolate *Dunaliella* sp. ABRIINW-S1.5 was clustered with intron-harboring species of *D. bardawil*, *D. parva*, and *D. viridis*. The results indicated that Urmia Lake is composed of isolates with different *18S rDNA* profiles with various intron arrangement.

Keywords: *18S rDNA*, Halotolerant unicellular microalga, Molecular screening.

INTRODUCTION

Dunaliella is a halotolerant unicellular microalga which has exceptional ability in biosynthesis of β -carotene (up to 10% of dry weight) (Ben-Amotz and Avron, 1983). In addition to β -carotene, *Dunaliella* strains can accumulate glycerol in response to salinity stress (Ben-Amotz and Avron, 1990). In the recent years, *Dunaliella* has been suggested to possess prominent advantages in molecular farming (Barzegari *et al.*, 2009).

Saline waters, oceans, marshes and salty lakes are the preferred locations for existence of the microalga *Dunaliella* (Borowitzka and Borowitzka, 1988). Urmia Lake, as the second largest salt-water lake on earth located in Northwest Iran, accommodates one of the world's richest biodiversity. It is full of mysterious cryptic organisms including microalga *Dunaliella* (Eimanifar and Mohebbi, 2007). During the last years since 2000, the lake is shrinking significantly. The reduction in the lake water level may threaten biodiversity, aquatic ecosystems, and species interactions (Gaeta *et al.*, 2014). Considering the decline of this

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valuable genetic source as well as beneficial features of *Dunaliella*, isolation and identification of its endogenous strains is of high importance.

Many authors have suggested that due to lack of morphological standard characteristics discrimination is difficult for most species of microalgae. Furthermore, physiological variability has been reported at the intraspecific level in *Dunaliella* (Gomez et al., 1999). To overcome biased physiological and morphological disadvantages, molecular features have been used to help to confirm the microalgal taxonomy. By employing molecular markers, a number of polyphyletic genera have been identified and the existence of genetically distinct lineages within single morpho-species has been revealed (Pröschold et al., 2001; Friedl and O'Kelly, 2002).

Identification and classification of the organisms based on conserved and variable regions is a common procedure in taxonomic studies (Hosseinzadeh et al., 2012). Thus, *18S rDNA* gene has been used for molecular identification of different species of *Dunaliella* as eukaryotic microorganism. The *18S rDNA* gene in *Dunaliella* genus contains relatively conserved region of exon(s) and possible variable region of intron(s).

The general purpose of the current study was to collect, isolate, and screen the molecular pattern of indigenous *Dunaliella* isolates based on *18S rDNA* gene in this important natural source.

MATERIALS AND METHODS

Sampling and Geographical Location

Urmia Lake, as the saltiest lake of the world after Dead Sea, and the biggest basin of Western Asia and also the most important lake of Iran, is located in the north west of Iran Plateau, between East and West Azerbaijan Provinces. This lake has a unique ecosystem with limited halophile species

such as a variety of green algae and cyanobacteria.

In total, 32 samples were taken from 8 different regions of the Urmia Lake and the origin names were described (Figure 1), covering East, North, Northeast, West (coast and deeper parts) areas, each area with at least 4 replications.

Preparation and Culturing of *Dunaliella* Samples

Electrical Conductivity (EC) of the samples was measured in the lab, using an EC meter (Cond 315i). Liquid medium was prepared by the method described by Hejazi et al. (2010) containing 90 g L⁻¹ NaCl. The samples were inoculated with prepared medium (1: 9) and maintained in temperature of 24°C and irradiance of 80 μmol photon m⁻² s⁻¹ in the photoperiod of 16: 8 (L:D).

To obtain single colonies, 5 μL of the liquid medium was spread on solid medium (1.8% agar). They were cultured in the previously described condition. After appearing single colonies on the media, these colonies were again transferred to liquid medium. As standard known samples, the standard strain of *D. salina* 19/18 was obtained from a Culture Collection of Algae and Protozoa (CCAP). It was cultivated under the same condition, as well.

DNA Extraction

DNA extraction of populations as well as single colonies was performed by the method described by Hejazi et al. (2010).

PCR amplification

Polymerase Chain Reactions (PCR) were carried out by conserved and species-specific primers. Conserved primers were designed according to 3' and 5' termini of *18S rDNA* regions by Olmos et al. (2000).

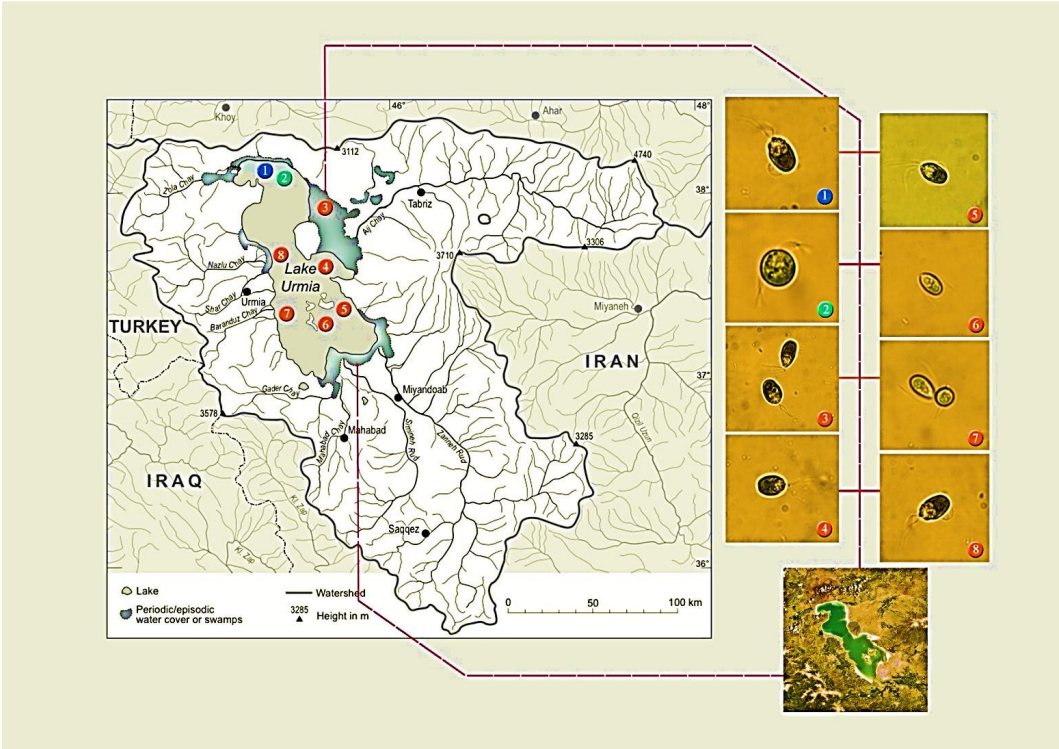


Figure 1. Geographical origins of Urmia Lake, West Azerbaijan Province, from where the samples were taken. Sampling regions of *Dunaliella* species are indicated by numbers. (1) Area Near Tasouj; (2) Sharafkhaneh Port; (3) Tabriz Plain; (4) Aji Chay Estuary; (5) Rahmanlou Port; (6): Barandouz Chay Estuary; (7) South central Water, and (8) Nazlu Chay Estuary.

They include MA1: [5'-CGGGATCCGTAGTCATATGCTTGTCTC-3'], MA2: [5'-CGGAATTCCTTCTGCAGGTCACC-3'].

For identification of the isolates at species level, other primers were designed based on the sequences of introns in different species (Olmos *et al.*, 2000; Olmos-Soto *et al.*, 2002). They were used together with MA2 primer as reverse primer. These specific primers include DSs, DP and DBs. These primers could amplify the intron within *18S rDNA* in *D. salina* and *D. parva* (Wilcox *et al.*, 1992) and *D. bardawil* (Olmos *et al.*, 2000). Using Oligo 5, another pair of primers was designed based on the intron existing in new *18S rDNA* arrangement reported by Hejazi *et al.* (2010). These primers were called as DMf [5'-ATGATAGGGGCAGTGAAAAGC-3'] and DMr [5'-CAGCAGGGCTCGACTATATC-3'].

PCR was performed for both specific and conserved primers with 25 μ L of sample containing 25 ng of genomic DNA dissolved and 50 ng of each primer. The amplification conditions for these primers (except DMf-DMr), were according to those proposed by Olmos *et al.* (2000). The amplification condition regarding the primers DMf-DMr was considered as follows. The initial denaturation of 4 minutes at 94°C, 32 amplification cycles consisting of 1 minute at 94°C for denaturation, 35 seconds at 64°C for annealing and 30 seconds at 72°C for extension and final extension of 10 minutes at 72°C.

Purification and Sequencing of PCR Products

To get a deep identification, *18S rDNA* gene sequencing of five isolates was performed.



Four of the isolates were selected from the group containing *18S rDNA* fragment of 1,770 bp and the remaining isolate belonged to the group with *18S rDNA* size of 2,170. These isolates were named as *Dunaliella* sp. ABRINW-Ch5, Ch3.1, Sh6-3, U1/1 and S1-5. For this purpose, the PCR products were purified according to the manufacturer's instructions described in PCR Purification Kit (Roche, Product No. 1732668). The purified amplicon was sent to Macrogen Company (Korea) for sequencing.

Alignment and Phylogenetic Analysis of Sequences

Sequence alignment and phylogenetic analyses of *18S rDNA* sequence were carried out using MAFFT online software available at <http://mafft.cbrc.jp/alignment/software/>. In this regard, the *18S rDNA* sequences of different *Dunaliella* strains were collected from NCBI. *Chlamydomonas reinhardtii* was determined as out group.

RESULTS AND DISCUSSION

Sampling and Isolation

Salinity measurement of the samples collected from different regions of Urmia Lake (Figure 1) showed that the highest salinity was related to the north and northeast of the lake (300- 350 g. L⁻¹ of salt). Salinity of eastern and western coasts was as 280 g L⁻¹ and 180 g L⁻¹, respectively. The total average of the samples was estimated at about 266.4 g L⁻¹. Differences in salinity levels were estimated to influence biodiversity profile of *Dunaliella* in various regions of the lake.

Amplification of *18S rDNA* Region

Using conserved primers, three 1,770, 2,170, and 2,550 bp fragments were amplified (Figure 2-a) as *18S rDNA* region. This preliminarily showed that there is *18S rDNA* variability in different *Dunaliella* populations.

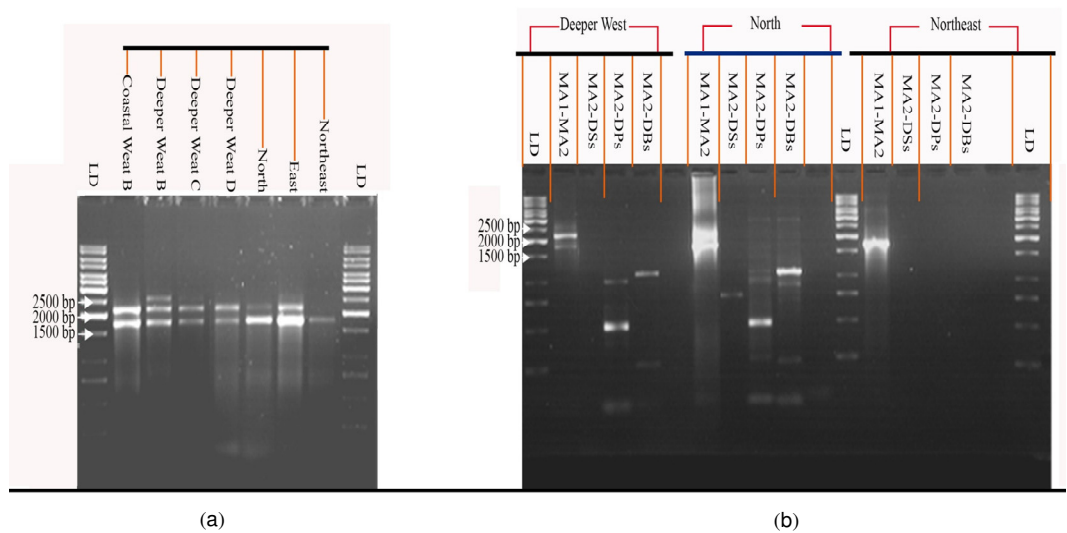


Figure2: Urmia Lake, from left to right respectively. (B): Amplified *18S rDNA* with conserved and specific primers, in the population of deeper west, north and northeast of Urmia Lake, with MA1-MA2, MA2-DSs, MA2-DPs and MA2-DBs from left to right respectively. The molecular weight marker was 1Kb ladder

In the populations related to the coastal and deeper parts located in the west of Urmia Lake, different varieties of fragments (1,770, 2,170, and 2,570 bp) were observed. In the coastal region, fragments of 1,770 and 2,170 bp were obtained, whereas in the deeper parts all three fragments existed. The molecular variation present in the northern parts was the same as deeper parts of western regions with three bands, while in the northeast only one fragment (1,770 bp) was amplified. The eastern regions of the lake showed less variation, which was similar to the coastal parts in the western regions. Two fragments with sizes of 1,770 and 2,170 bp were obtained in this part. Each of these *18S rDNA* fragment sizes showed us an initial indication of certain species. According to Olmos *et al.* (2000), *18S rDNA* of *D. salina* has a band of 2,170 bp and contains one intron (near 5' terminus), while this region in *D. parva* is 2,570 bp with two introns. On the other hand, *18S rDNA* of *D. tertiolecta* (Olmos *et al.*, 2000) is 1,770 bp without any intron, and it is 2,570 bp in *D. bardawil* containing two introns. Furthermore, according to the submitted sequence of *D. viridis* (Gonzalez *et al.*, 1999) with accession number of DQ009776 at GenBank, using these primers, *18S rDNA* size of about 2,500 bp is expected. Prior to the present study, our group verified and introduced a novel strain of *Dunaliella* sp. from Maharlou, a saline lake in Iran, namely, *Dunaliella* sp. ABRIINW-M1/2 with *18S rDNA* of 2,170 bp, which harbored one intron near 3' terminus (Hejazi *et al.*, 2010).

To study at species level, PCR was performed using species-specific primers (Figure 2-b). Primer pair of MA2-DSs was amplified in a few samples of the population. It produced the desired fragments only in the samples of the north and east of the lake. Whilst, using MA2-DPs and MA2-DBs potent fragments were amplified in the samples. The pairs MA2-DPs and MA2-DBs were well amplified in the samples collected from all regions of the lake, except the northeast region. In

addition, DMr and DMf were amplified in the desired fragments of 250 bp in the samples of north and east of the lake.

According to Olmos *et al.* (2000 and 2002), by amplification with species-specific primers of DSs, DPs, and DBs, three species of *D. salina*, *D. parva* and *D. bardawil* are respectively discernible. Using DSs-MA2, a fragment of ~750 bp in *D. salina*, using DPs-MA2, a fragment of ~1,050 bp in *D. parva*, and using DBs-MA2, a fragment of ~1,000 bp was amplified in *D. bardawil*. The latter may produce a band of ~500 bp with DPs-MA2. Because *18S rDNA* of *D. tertiolecta* does not harbor any intron, no amplification with species-specific primers occurs. The early variability pattern in the populations predicted existence of *D. bardawil*, *D. parva*, *D. salina*, *D. tertiolecta* (Olmos *et al.*, 2000; Olmos-Soto, *et al.*, 2002) and isolates similar to *Dunaliella* sp. ABRIINW-M1/2 in Urmia Lake (Hejazi *et al.*, 2010). These observations confirmed the existence of different *Dunaliella* species in Urmia Lake.

Furthermore, these results suggested that the genetic variation in *18S rDNA* region of *Dunaliella* was different depending on the various parts of the lake. Logically, the reason for different series of isolates might be due to salinity difference in various regions of the lake. The finding is in agreement with the results showing that different *Dunaliella* species have a salinity preference for their optimum growth reported by Borowitzka and Christopher (2007).

Amplification of *18S rDNA* Region in Single Colonies

For single colonies, PCR using MA1-MA2 primers led to production of fragments with 1,770, 2,170, and 2,550 bp sizes. In the next step based on *18S rDNA* size of each isolate, DNA amplification by species-specific primers was performed (Figures 3-a, -b, -c and -d).

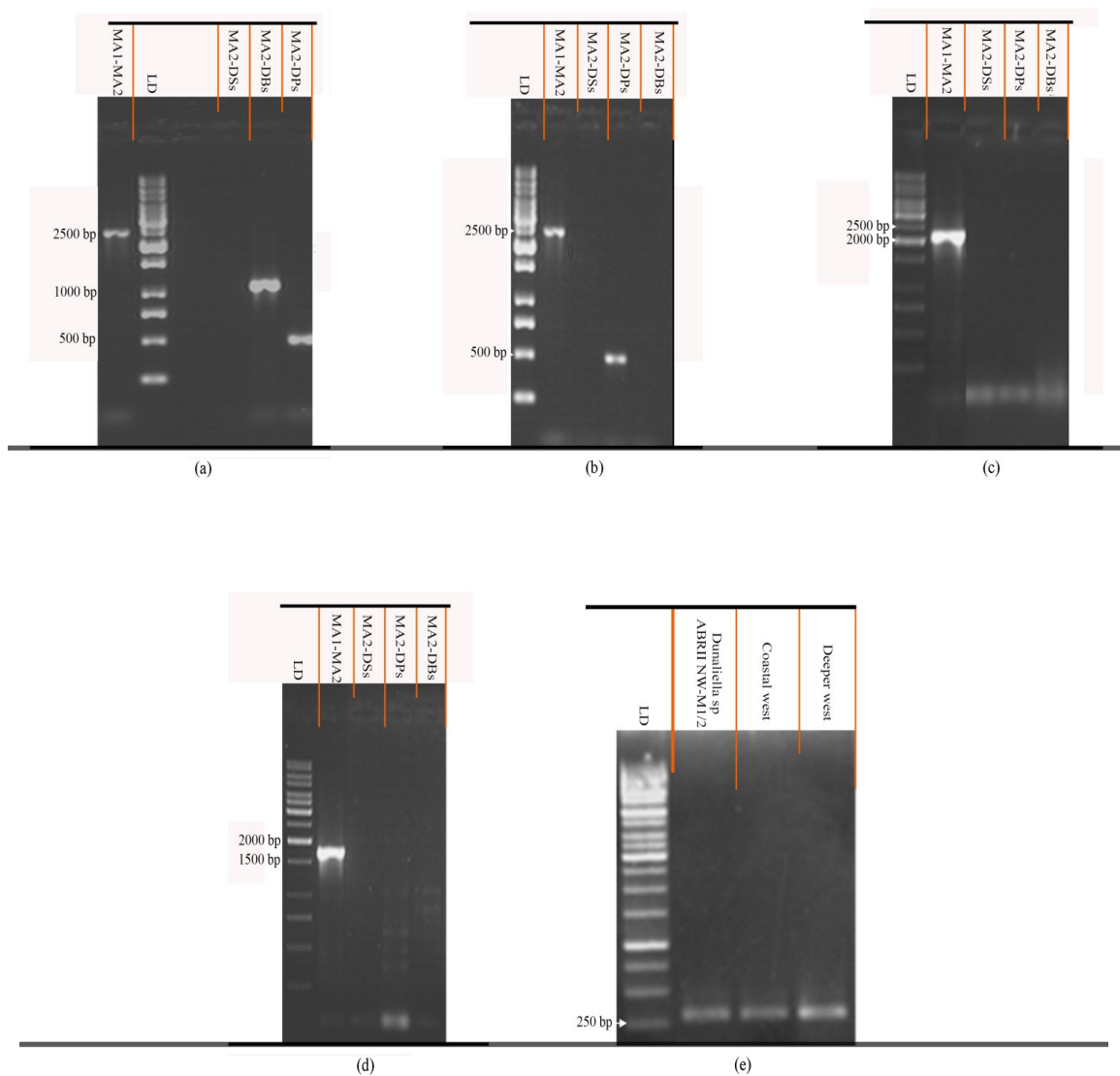


Figure 3. (a) The isolate producing *18S rDNA* amplified fragment of 2550 bp with MA1-MA2, 500 bp with MA2-DPs and 1000 bp with MA2-DBs. (b) The *Dunaliella* isolate producing 18SrDNA amplified fragment of 2550 bp with MA1-MA2 and 500 bp with MA2-DPs. (c) The isolate producing *18S rDNA* amplified fragment of 2150 bp with MA1-MA2 and no fragment with MA2-DSs. (d) The isolate producing *18S rDNA* amplified fragment of 1770 bp with MA1-MA2 and no fragment with MA2-DBs, MA2-DPs and MA2-DSs. (e) Amplified *18S rDNA* with specific primers DMf-DMr on 3 single colonies, including *Dunaliella* sp. ABRIIW-M1/2, Coastal West and deeper west.

All the obtained single colonies were successfully amplified with MA1-MA2, confirming the genus of the isolates as *Dunaliella*. The isolates, in which a fragment of ~ 1,000 bp with MA2-DBs and a 500 bp fragment with MA2-DPs were

produced, were attributed to *D. bardawil*. For the isolates with *18S rDNA* gene of 2,570 bp and a fragment of 500 bp produced with DPs-MA2, no certain species were determined. The colonies with *18S rDNA* of 2,170 bp were divided into two groups: in

one group ~250 bp was amplified with specific primers of DMf-DMr. These were recognized as relatives of the described taxon, *Dunaliella* sp. ABRIINW-M1/2. The other group did not produce any amplicon with species-specific primers and we could not attribute them to any of the known species. The *18S rDNA* gene of 1,770 bp and presence of no intron led to identification of the majority of the isolates (16 isolates) as *D. tertiolecta*.

Table 1 illustrates the fragment size of amplified *18S rDNA* for each isolate obtained. According to the data shown, out of thirty two isolates, seven isolates produced *18S rDNA* size of 2,550 bp. Out of these, four produced ~1,000 bp fragments with MA2-DBs and 500 bp fragments with MA2-DPs. Three other isolates produced a fragment of 500 bp with MA2-DPs.

Subsequently, PCR was performed for nine isolates with *18S rDNA* gene of 2,170 bp using MA2-DSs and DMf-DMr primers. They all produced no fragments with MA2-DSs. Only two isolates produced a fragment of 250 bp with DMf-DMr (Figure 3-e). These isolates were comparable with *Dunaliella* sp. ABRIINW-M1/2 (Hejazi *et al.*, 2010) from which the primer pair was designed and the same fragment size was amplified with them. The remaining seven isolates produced no fragment with DMf-DMr unabling us to relate them to any species.

Sixteen isolates produced 1,770 bp as *18S rDNA*. No amplification was observed with species-specific primers in these isolates. It

should be considered that intron-sizing method developed by Olmos *et al.* (2000 and 2002) is rapid and sensitive, for specifically identifying species of *Dunaliella*. However, according to this method we were not able to imbed some of our isolates within certain species.

To have a deep insight into gene sequence of *18S rDNA* in our isolates, five isolates (four from the group containing 1,770 bp and one from 2,170 bp *18S rDNA* gene) were sequenced and compared with the known registered species. In the *18S rDNA* region, an extensive variable site was detectable extending from 1,093-1,919 of the consensus sequence (Figure 4). It is related to the first intron of *D. parva* and *D. bardawil* and the only intron of *D. salina* and *D. viridis*. Our isolated *Dunaliella* members all lacked this region. The second variable region starts from 2,494 and relates to second intron of *D. parva*, *D. bardawil* and only intron of *Dunaliella* sp. ABRIINW-M1/2. The comparative alignment analysis showed that only *Dunaliella* sp. ABRIINW-S1.5 harbors the variable region (related to intron) in the 3' terminus. This intron position is similar to the one reported for *Dunaliella* sp. ABRIINW-M1/2. However, sequence composition similarity of the intron was not confirmed by phylogenetic tree. This finding confirmed pattern variability of *18S rDNA* intron.

The phylogenetic tree of the indigenous and the species described based on Neighbor-joining method is depicted in Figure 5. As expected, *Chlamydomonas reinhardtii* appeared as outgroup. According to the tree, the isolates *Dunaliella* sp.

Table1. *18S rDNA* amplification results of *Dunaliella* isolates with conserved and species- specific primers.

PCR size	N ^a	Produced bands with specific primers (bp)				Identifies species
		MA2-DSs	MA2-DPs	MA2-DBs	DMf-DMr	
2570 bp	4	*	500 bp	~1000 bp	*	<i>D. bardawil</i>
	3	*	500 bp	No band	*	?
2170 bp	7	No band	*	*	No band	?
	2	No band	*	*	250	<i>Dunaliella</i> sp. ABRIINW-M1/2
1770 bp	16	*	*	*	*	<i>D. tertiolecta</i>

^a The number of isolates, PCR size: Size of *18S rDNA* (bp).



Figure 4. 18S rDNA sequence alignment of Urmia *Dunaliella* isolates and other species. The variable regions of the gene are demonstrated in different color. The diagram shows the position of intron insertions of 18S rDNA gene.

ABRIINW-Ch5, Sh6.3 and U1/1 were grouped within a clade containing the species *D. salina*, *D. tertiolecta*, *D. bioculata*, *D. primolecta*, and *D. peircei*.

Dunaliella sp. ABRIINW-Ch3.1 was clustered with *Dunaliella* sp. ABRIINW-M1/2, while the isolate *Dunaliella* sp. ABRIINW-S1.5 was clustered with the



Figure 5. Phylogram based on *18S rDNA* gene for *Dunaliella* species and indigenous isolates from Urmia Lake. The tree is constructed using MAFFT, Neighbor-joining method. The sequenced native isolates are shown in rectangles.

species *D. bardawil*, *D. parva* and *D. viridis*. The sequences of *Dunaliella* sp. ABRIINW-S1.5, Ch3.1, Ch5, Sh6.3 and U1/1 were submitted as JQ922245, JQ694056, JQ694055, JQ712983 and FJ164062.

CONCLUSIONS

In this study, we applied intron-sizing method to compare the *18S rDNA* fingerprint between *Dunaliella* isolates of Urmia Lake. According to the fragment size of *18S rDNA*, we observed the genetic variation in *Dunaliella* genus depending on different areas of Urmia Lake. PCR with species-specific primers in the population confirmed the existence of at least four species of *D. tertiolecta*, *D.*

parva, *D. salina*, and *D. bardawil* and some types of isolates which were similar to *Dunaliella* sp. ABRIINW-M1/2. Studies of single colonies indicated that we were able to isolate and identify some isolates belonging to *D. bardawil*, *D. tertiolecta*, and the isolates similar to *Dunaliella* sp. ABRIINW-M1/2. In summary, this study indicated that our method was appropriate to differentiate between some species of *Dunaliella* and rapidly identify them. However, more conserved genes such as ITS for identification of *Dunaliella* isolates in Urmia Lake need to be evaluated before any confirmation of high potential carotenogenic indigenous strains may be considered for use in commercial selection programs.



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حفاظت و آنالیز تنوع زیستی ریز جلبک دونالیا در دریاچه شور ارومیه براساس روش اندازه گیری اینترونی

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چکیده

دریاچه ارومیه دومین دریاچه شور جهان است که منبع اصلی ریزجلبک تک سلولی دونالیا (*Dunaliella*) در ایران به شمار می رود. در سال های اخیر این دریاچه و در نتیجه تنوع ژنتیکی آن از نظر محیطی مورد تهدید قرار گرفته است. از این رو جمع آوری، حفظ و شناسایی ریزموجودات این دریاچه اهمیت بالایی دارد. هدف از این تحقیق غربال مولکولی جدایه های دونالیا در دریاچه ارومیه است. به این منظور، ۳۲ نمونه از مناطق جغرافیایی مختلف دریاچه جمع آوری شد. سپس الگوی مولکولی نمونه ها بر پایه ژن *18 S rDNA* و روش اندازه گیری اینترونی (intron-sizing) بررسی شد. نتایج براساس آغازگرهای حفاظت شده و اختصاصی گونه، نشان داد که بسته به نواحی مختلف دریاچه، تفاوت در تنوع ژنتیکی جمعیت دونالیا وجود دارد. الگوی تکثیر شده برای ایزوله های منفرد مشابه الگوی گونه های شناخته شده *D. tertiolecta*، *D. bardawil* و *Dunaliella sp.* بود. توالی یابی و تجزیه و تحلیل فیلوژنتیکی ناحیه *18 S rDNA* ی ۵ ایزوله شاخص نشان داد که ایزوله های *Dunaliella sp.* ABRIINW-Ch5، Sh6.3 و U1/1- با گونه های فاقد اینترون دونالیا هم گروه شدند. جدایه *Dunaliella sp.* ABRIINW-Ch3.1 با *Dunaliella sp.* ABRIINW-M1/2 هم خوشه شد. در حالیکه جدایه *Dunaliella sp.* ABRIINW-S1.5 با گونه های حاوی اینترون *D. bardawil*، *D. parva* و *D. viridis* هم گروه شد. نتایج نشان می دهد که دریاچه ارومیه مخلوطی از ایزوله های مختلف با پروفیل متفاوت *18 rDNA* و آرایش متنوعی از اینترون است.