Characterization of an Indigenous Isolate, *Dunaliella tertiolecta* ABRIINW-G3, from Gavkhooni Salt Marsh in Iran Based on Molecular and Some Morpho-physiological Attributes

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ABSTRACT

*Dunaliella* is a green halotolerant microalga, which has several industrial applications e.g. β-carotene production. Identification of different *Dunaliella* species has been carried out by morpho-physiological and recently molecular studies. To achieve an improved understanding of taxonomy, these studies are required to be in linkage. The present study describes molecular and specific morpho-physiological properties of a *Dunaliella* isolate obtained from Gavkhooni salt marsh in Iran. Phylogenetic analysis of Internal Transcribed Spacer region demonstrated that the isolate was associated with different species except *D. salina* (CCAP 19/18 and 19/30) and *D. viridis*. 18S rDNA size of the isolate was identical to that of *D. tertiolecta* and intron-lacking strains of *D. salina*. 18S rDNA fingerprint profile and phylogenetic analysis revealed *D. tertiolecta* as the closest taxon to the isolate. Features of optimum growth salinity (1.5-3% w/v) and maximum carotenoid per cell (0.7 pg cell⁻¹) were comparable with reported data for *D. terrtiolecta*. Morphological characteristics including the size and color of the cells, presence and location of stigma and refractile granules were similar to those of *D. tertiolecta*. Totally, considering molecular and morpho-physiological properties, the isolate was attributed to the species *D. tertiolecta* and was named as *Dunaliella tertiolecta* ABRIINW-G3.

Keywords: Carotenoid, *Dunaliella*, 18S rDNA, Internal Transcribed Spacer, Optimum growth salinity.

INTRODUCTION

The green alga *Dunaliella* is a unicellular, eukaryotic and photosynthetic microorganism that lacks a rigid cell wall (Ben-Amotz and Avron, 1987). As one of the main sources of natural β-carotene, *Dunaliella* has attracted growing interest in nutraceutical, cosmetic and pharmaceutical industries (Hejazi et al., 2002). Further, because *Dunaliella* represents unique biological and technical features, it has been the subject of study in molecular farming (Barzegari et al., 2010). *Dunaliella* can adapt to a wide range of salt concentrations varying from low (0.05M) to saturation (5.5M) level (Ben-Amotz and Avron, 1973; Borowitzka and Brown, 1974). In addition, it can survive high light intensities (Ben-Amotz and Avron, 1982; Borowitzka et al., 1984) due to carotenoid accumulation ability, which protects *Dunaliella* against intense irradiation (Ben-Amotz et al., 1989; Gómez et al., 1992). Under stress conditions such as elevated light densities, high salinity and nutrient deficiency,
ß-carotene accumulation may reach a level more than 10% dry weight of *Dunaliella* (Ben-Amotz and Avron, 1983; Ben-Amotz, 1995). Commercial considerations and achieving effective classification require increasing attention to isolation and delineation of various strains of *Dunaliella*. Different species of *Dunaliella* have been identified based on morphological characteristics and physiological behaviour. However, lack of a rigid cell wall and presence of compensatory adaptation processes in the members of *Dunaliella* (Sciandra et al., 1997) lead to variable morpho-physiological features. Consequently, misidentifications and confusions are possible in the taxonomy of *Dunaliella*. Molecular techniques may provide a powerful tool in distinguishing different species (Gómez and González, 2004; Olmos et al., 2000, 2009). In order to better understand the taxonomy of the genus, molecular investigations are recommended to accompany morphological/physiological studies (Borowitzka and Siva, 2007) as was observed in the recent research by Hejazi et al. (2010) and Azua-Bustos et al. (2010). Analysis of ribosomal spacer sequences, including internal transcribed spacer region (ITS1 and ITS2 plus the 5.8S rDNA gene) has been carried out for phylogenetic studies in *Dunaliella* genus (González et al., 1999; González et al., 2001; Gómez and González, 2001, 2004). Further, molecular markers based on 18S rDNA gene have been used for the identification of *Dunaliella* species (Olmos et al., 2000, 2002; Fazeli et al., 2006; Raja et al., 2007; Hejazi et al., 2010). Using 18S rDNA region, four different structures within the genus of *Dunaliella* have already been described (Olmos et al., 2000, 2002; Hejazi et al., 2010): the two structures which contain one intron but in two different positions, the structure that has two introns, and the structure that lacks any intron in 18S rDNA gene. The latter is reported in the species of *D. tertiolecta* and some strains of *D. salina* (e.g. UTEX LB1644).

Having several salt lakes and marshes, Iran can be considered as an appropriate habitat for microalga *Dunaliella*. In this study, an indigenous isolate was obtained from Gavkhooni salt marsh in Iran. The isolate was described based on the genomic regions of 18S rDNA and ITS. Cell morphology as well as the physiological features of optimum growth salinity and maximum cellular carotenoid were determined.

**MATERIALS AND METHODS**

**Isolation and Cultivation**

Water sample was collected from Gavkhooni salt marsh in Iran and was then inoculated with a liquid medium described by Hejazi and Wijffles (2003). After several recultivations, the cultured specimen was spread on the same medium prepared by 1.8% agar. An individual isolate was selected from the culture for the experiment. The cultured isolate was maintained at 26°C and a light intensity of 80 µmol photon m⁻² s⁻¹ under the light to dark photoperiod of 16:8 hours.

**DNA Extraction and PCR Amplification of ITS Region and 18S rDNA Gene**

Isolation of genomic DNA was carried out by the method suggested by Hejazi et al. (2010). ITS region was amplified using two primers of AB1 (5'-AATCTATCAATAACCACCG-3') and AB2 (5'-TTTCATTCGCCATTACTAAGG-3'). Using Oligo5, these primers were designed from the nucleotides 1-21 of ITS1 and 80-100 of 28S rDNA gene, respectively. PCR reactions were performed in 50 µl volumes containing 20 ng of template DNA in TE (Tris/EDTA) buffer and 50 ng of each primer using 1X PCR Master Kit (CinnaGen PCR Master Kit, Cat. No. PR8250C). The amplification was performed in 35 cycles using a TECHNE Thermal Cycler (Model: FTGRAD2D) according to the method described by Hejazi et al. (2010).
18S rDNA amplification was performed using two conserved primers called MA1 and MA2 (Olmos et al., 2000). To delineate at species level, three species-specific primers of DSs, DBs and DPs were utilized; DSs, DPs and DBs were designed (Olmos et al., 2000, 2002) according to the single intron of D. salina, the first intron of D. parva and D. bardawil, respectively. They were used as forward primers along with the reverse primer of MA2. Thermocycling consisted of 5 minutes initial denaturing time at 95ºC, followed by 35 cycles at 95ºC for 1 minute, 52 ºC for 1 minute and 72ºC for 2 minutes with final extension of 72 ºC for 10 min.

**Alignment and Phylogenetic Analysis of Sequences**

ITS and 18S rDNA sequence of several strains (Table 1) including *Chlamydomonas reinhardtii* as outgroup was collected from National Center for Biotechnology Information (NCBI). The sequence alignment was performed using MEGA version 4 (Tamura et al., 2007). The alignment statistics were estimated by ‘DNA Sequence Polymorphism’ (DnaSP), version 5 (Librado and Rozas, 2009). Phylogenetic analyses were conducted using Neighbour-Joining method. The evolutionary distances were computed by the Maximum Composite Likelihood model. Reliability of the branches was assessed by bootstrapping the data with 1,000 replicates.

**Purification and Sequencing of PCR Products**

Purification was performed according to the manufacturer's instructions described in the PCR purification kit (Roche, Product No. 1732668). The purified amplicon was sent to Macrogen Company (Seoul, Korea) for sequencing.

**Determination of Cellular growth and Carotenoid Production**

In order to determine the optimum growth salinity and carotenoid content of the isolate, the media were prepared with 7 salt concentrations of 0.25, 0.5, 1, 1.5, 2,
2.5 and 3 M NaCl each in 3 replications. The cultures were maintained at an irradiance of 80 μmol photon m\(^{-2}\) s\(^{-1}\). Cell counting was performed every other day using Neubauer counting chamber (HBG, Germany, Tiefe depth profondeur 0.10 mm and 0.0025 mm\(^2\) area). Carotenoid content of the cells was determined once every four days. For carotenoid extraction, the following procedure was used; 2 ml of the culture was centrifuged (Centrifuge Beckman, Allegra X-22R, USA) at 948 xg for 10 minutes. Supernatant was thrown away and 5 ml of KOH (5%) dissolved in methanol (30%) was added to the biomass. The sample was maintained at 60°C for 20 minutes to remove chlorophyll. Then the container was centrifuged at 2132 xg for 10 minutes. The upper fluid was discarded. To the remaining biomass of yellow/orange-color, 2 ml of Hexane-Acetone (1:9) was added and the tubes were well spun. Finally, after 5 min centrifugation at 658 xg, carotenoid concentrations were determined using a spectrophotometer (Genesys 5, Model 336001, USA). Total carotenoid content was calculated according to the equation obtained by calibration curve (Hejazi and Wijffels, 2003) using the following formula:

\[
C = A_{446} \times 3.26 \times V_C / V_S
\]

Where, \(C\) = Total carotenoid (μg ml\(^{-1}\)), \(V_c\) = Volume of culture sample and \(V_s\) = Volume of extract (ml).

The sample absorbance was scanned over the wavelengths of 350-800 nm and \(\lambda_{max}\) was recorded at 446 nm.

**Statistical Analysis**

The data comparison for different salinity levels was performed based on Completely Randomized Design (CRD) method using SAS software (Release 6.12, SAS Institute Inc., Cary, NC, USA). Means were separated by the Duncan’s multiple range test (Duncan, 1995).

**Light Microscopy**

Morphological characteristics including the shape and size of the cells, absence/presence and location of stigma and refractile granules were examined using a light microscope (Zeiss, Axiostar Plus, Germany). To observe pyrenoids, the cells were treated with 0.05% bromophenol blue (BPB) dissolved in 0.1% HgCl\(_2\) (Brown and Arnott, 1970).

**RESULTS**

**Amplification and Sequence Analysis of ITS Region**

A fragment of ~700 bp was amplified as ITS region with primer pair (AB1-AB2) specific for ITS region of *Dunaliella*. The amplification confirmed the isolate as a member of *Dunaliella* genus. To achieve ITS sequence composition, the amplified region was sequenced. Using the software “Basic Local Alignment Search Tool” (BLAST) at NCBI, the sequence of the isolate was compared with other recorded data. It exhibited various similarities of 99% to *D. tertiolecta* CCAP 19/27, *D. salina* SAG 42.88, *D. tertiolecta* SAG 13.86, *D. primolecta*, *D. bardawil*, *D. parva*, 94% to *D. salina* CCAP 19/18 and 92% to *D. salina* CCAP 19/30. During the next analyses, the isolate of our interest was coded as *Dunaliella* sp. ABR1N1W-G3. To analyze the ITS region and establish a phylogenetic tree, the sequences of different strains given in Table 1 were utilized. Because analysis of the entire ITS region (including 5.8S rDNA) was aimed, the sequences of the six following strains were excluded: *D. salina* M84320, *D. bardawil* LB2538 and *D. atacamensis* for which there was no recorded ITS sequence; *D. salina* UTEX LB 1644, *D. tertiolecta* CCMP 1320 and *D. tertiolecta* UTEX LB 999 for which ITS regions were registered as separate ITS1 and ITS2 sequences, lacking 5.8S rDNA gene, at NCBI. After alignment, using DnaSP
software, the sequence conservation was determined as C: 0.619 and polymorphic sites were calculated as 228 out of 598 nucleotides. The tree (Figure 1) illustrated that *Ch. reinhardtii* was the most divergent from all other analyzed *Dunaliella* taxa. The 14 sequences belonging to *Dunaliella* members were divided into three lineages of a, b and c. *Dunaliella* sp. ABRIINW-G3 appeared as an individual entity which was grouped (Bootstrap value: 96%) with the clade containing *D. tertiolecta* strains ‘CCMP 1302’, SAG 13.86 and CCAP 19/27, *D. primolecta* UTEX LB 1000, *D. bioculata* UTEX LB 199, *D. salina* SAG 42.88 and *D. parva*. The members of this clade showed the least evolutionary distance (0.009) with the isolate. Evolutionary distance, as a measure of evolutionary divergence, is the number of substitutions per site that have occurred between a pair of homologous sequences. The second lineage (b) contained *Dunaliella* sp. ABRIINW-M1/1, *D. salina* CCAP 19/18, *D. salina* CCAP 19/30, *Dunaliella* sp. ABRIINW-U1/1 and *D. salina* MSI-1. Finally *Dunaliella* sp. ABRIINW-M1/2 and *D. viridis* were included within the third lineage (c). The ITS sequence of *Dunaliella* sp. ABRIINW-G3 was registered at NCBI database with accession number of HQ590542.

**Amplification and Sequence Analysis of 18S rDNA Gene**

Amplification of 18S rDNA region with MA1-MA2 resulted in the production of a ~1770 bp DNA fragment. Using three species-specific primers of DSs, DPs and DBs (Olmos *et al.*, 2000, 2002), no fragment was produced.

To obtain the sequence fingerprint profile of the 18S rDNA, sequencing was performed. Then the obtained sequence was aligned with 18S rDNA sequences related to other known species (Table 1). Out of 1,702

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**Figure 1.** Phylogenetic tree based on ITS region of various strains of *Dunaliella* using neighbor-joining method. Bootstrap values, as measures of supporting a given grouping, were calculated from 1,000 replicates. Bar represents 0.05 nucleotide substitutions per position. Three lineages of a, b and c were produced. Within lineage a, *Dunaliella* sp. ABRIINW-G3 was grouped with different members of *Dunaliella*. *D. salina* MSI-1 isolated from Maharlou Lake was included in Lineage b.
net number of analyzed sequence sites, 14 polymorphic sites and a high conserved region extending from 33 to 1,645 nucleotides were determined. Sequence conservation was estimated to be 0.992. According to the phylogenetic tree (Figure 2), five lineages of A, B, C, D and E were supported with high bootstrap value (more than 80%). As an outgroup, Ch. reinhardtii appeared in a distinctive branch. The isolate of our interest, was located in lineage B which contained D. tertiolecta CCMP 1302, D. tertiolecta UTEX LB 999, D. viridis and Dunaliella sp. ABRIINW-M1/2. Based on the evolutionary analysis (data not shown), D. tertiolecta CCMP 1302, with evolutionary distance of 0.002, was identified as the closest taxon to Dunaliella sp. ABRIINW-G3. The sequence of our isolate was registered at NCBI database with accession number of GU984571.

**Optimum Growth Salinity and Maximum Carotenoid Content**

To measure the optimum growth salinity, the starting Optical Density of inoculums in
all 7 salinity levels was adjusted to the same quantity of ~0.08 at 550 nm. Because there was not any significant growth at 2.5 and 3 M NaCl, these measurements were excluded from subsequent investigations. The data revealed that cell growth was significantly affected by NaCl concentration (P< 0.01). 26 days after inoculation, the isolate attained maximum cell density of 16.4×10⁶ and 15.83×10⁶ cell ml⁻¹ at 0.25 and 0.5 M NaCl, respectively (Figure 3). In contrast, the lowest cell density was determined at 2 M NaCl. The maximum carotenoid content was measured as 0.7 pg cell⁻¹ at 2 M NaCl, which was significantly higher than the other salinities (P< 0.01). During the absorbance scanning procedure (Figure 4), the main carotenoid type was assessed as β-carotene.

Cell Morphology

Morphological schema of the cells in 10 day old cultures of different salinities is depicted in Figure 5 (a-f). At different salt concentrations, the cells color was constantly green. However, cell shape changed varying from ellipsoidal at low NaCl levels of 0.25-1.5 M to spherical at the salinities of 2-3 M. The cells lack any cell wall and have one stigma (Figure 5-g) near the location where two equal flagella are inserted. Cells contain a cup-shaped chloroplast with a basal pyrenoid surrounded by starch granules (Figure 5-h). The lateral lobes of the chloroplast are not extended to the anterior end of the cell, which contained small refractile granules (Figure 5-h). The cell size was calculated by averaging the sizes measured at optimum growth salinity. The average cell length was about 11.6 μm with the range of 9 to 15 μm. The average width of the cells was 7.2 μm ranging from 5 to 9.5 μm. The average size for two equal flagella, inserted apically, was about 12.5 μm. Proportion of flagella length to cell length was ~ 1.1 (based on average sizes).

DISCUSSION

To date, identification of Dunaliella species/strains has already been the subject of morpho-physiological and recently molecular studies. However, these studies were mostly performed separately and led to ambiguity in achieving a thorough understanding of Dunaliella taxonomy. In 2007, Borowitzka and Siva illustrated the
deficiencies of this separation, emphasizing that these studies need to be paired. Iran has different salt lakes and marshes in which Dunaliella can grow. However, very few studies have been performed on native isolates of Dunaliella (Fazeli et al., 2006; Hadi et al., 2008; Zamani et al., 2011) and there is still demanding need to isolate and characterize the Iranian isolates of this microalgae. In this regard, this research was performed to identify and describe an indigenous isolate from Gavkhooni. Gavkhooni is a salt marsh located in the center of Iran where Dunaliella exists.

Figure 4. Absorption spectrum of the carotenoid extracted from 12-day old culture of Dunaliella sp. ABRINNW-G3.

Figure 5. Light micrographs of Dunaliella sp. ABRINNW-G3 in 10 day old cultures: a-f (Scale= 10 µm) show cell morphology at different salinity levels (a: 0.5; b: 1; c: 1.5; d: 2; e: 2.5, and f: 3 M NaCl), g and h are of the sections; Chl: Chloroplast; Pyr: Pyrenoid; St: Stigma; F: Flagella; S.G.: Starch grains, and R.G.: refractile granules.
Previously, two species of *D. salina* and *D. viridis* had been identified from Gavkhooni (Hadi et al., 2008). The salinity and pH of the collected water sample were respectively in the range of 0.5-2M NaCl and 7-8. The studied isolate was obtained from a water sample with salinity close to 0.5M and pH equal to 7.5. The ITS region size (~ 700 bp) of the isolate was identical to the sizes reported for different *Dunaliella* species. It was demonstrated that there was no ITS length variation at intra- or interspecific level of *Dunaliella* (González et al., 1999). Based on phylogenetic analysis of ITS region, *Dunaliella sp*. ABRII NW-G3 was grouped with the clade containing *D. tertiolecta*, *D. primolecta*, *D. bicuculata*, *D. bardawil*, *D. parva* and intron-lacking strains of *D. salina*. The mentioned different species within this clade were not differentiated using phylogenetic analysis of ITS region. The previously reported isolate of *D. salina* MSI-1 from Maharlou lake in Iran (Zamani et al., 2011) was placed on a separate lineage and was grouped with *D. salina* strains 19/18 and 19/30. This Iranian isolate showed 92% homology with *Dunaliella sp*. ABRII NW-G3. From Iran, another strain, *D. tertiolecta* (DCCBC26) had been identified originating from Urmia Lake (Fazeli et al., 2006). Because only ITS2 region of this strain was available, it was compared with the corresponding region in our isolate. The comparison showed that the two isolates shared 98% nucleotide similarity with each other.

The amplification by MA1-MA2 primers, which allows amplification of nearly full length of 18S rDNA in *Dunaliella*, resulted in the production of ~1770 bp fragment. According to Olmos et al. (2000) an amplicon of the same size is amplified in *D. tertiolecta* as well as some strains of *D. salina* (e.g. LB1644). 18S rDNA of the mentioned size is reported to have no intron within the gene and hence no fragment is amplified using the species-specific primers. It should be noted that in other strains of *D. salina* (e.g. 19/3, 19/18, M84320), which contain one intron, a band of ~2170 bp is created. Thus, in the studied isolate 18S rDNA size and amplification of no band by species-specific primers confirmed the lack of intron(s) in 18S rDNA. According to the analysis of evolutionary distances, the studied isolate showed the closest distance with *D. tertiolecta* 1302.

Optimum growth of *Dunaliella sp*. ABRII NW-G3 occurred at 0.25-0.5 M (1.5-3% (w/v)) NaCl. For *D. tertiolecta* DCCBC26 (from Urmia Lake, Iran) growth preference was reported as 0.3-0.7 M NaCl (Fazeli et al., 2006). It has been specified that *D. tertiolecta* and *D. salina* can grow well at the salinities of no more than 6% and 20-25%, respectively (Borowitzka and Siva, 2007). Therefore, optimum growth salinity of the studied isolate was comparable with that of *D. tertiolecta*. The maximum carotenoid per cell (0.7 pg cell") was close to the quantity reported for *D. tertiolecta* CCMP 19/22. Maximum carotenoid per cell occurred at 2 M NaCl (12%) in which cell growth was limited. This was in congruence with the studies that claim although elevated salinity (here 2M NaCl) favors carotenoid biosynthesis, it represses the growth (Gómez et al., 2003; Jahnke and White, 2003).

Morphologically, the green color of the cells in all media containing 0.25-3 M NaCl was retained. However, cell shape changed with salinity of the cultures; when cultured at elevated salinities the ellipsoidal shape turned to rounded. This was in agreement with previous studies which had demonstrated that cell shape of a certain species often became spherical under unfavorable conditions (Borowitzka et al., 1984; Ben-Amotz et al., 2009). *Dunaliella sp*. ABRII NW-G3, matched in cell size with *D. tertiolecta* except for the proportion of flagella length/cell length (1.1) which was less than *D. tertiolecta* (1.5-2).

In conclusion, based on molecular investigations of 18S rDNA gene and ITS regions, low carotenoids content, growing well at low salinities and morphological properties, the studied isolate was attributed to the species *D. tertiolecta* and named as *Dunaliella tertiolecta* ABRII NW-G3.
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1. Introduction

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شناسایی ایزوله بومی Dunaliella tertiolecta ABRIINW-G3 براساس خواص مولکولی و برخی صفات مورفو-فیزیولوژیکی

چکیده

دانالیلا یک ریزگرگ سیز مقاوم به شوری است که کاربردهای تجاری محدودی نظر تولید بتاکاروتین دارد. شناسایی گونه‌های مختلف دانالیلا براساس مطالعات مورفو-فیزیولوژیکی و یاخته مطالعات مولکولی صورت گرفته است. برای دستیابی به درک صحیح از تاکسنومی دانالیلا، توصیه می‌گردد این مطالعات به طور همزمان در شناسایی به کار گرفته شوند. تحقیق حاضر مطالعات مولکولی و برخی خصوصیات مورفولو-فیزیولوژیکی ایزوله دانالیلای جدایی را از دریاچه گاوخوئی در ایران را توصیف می‌کند. مقایسه توانایی حور فاصله بین نواحی رونویسی شونده (ITS) رابطزدیک این جداچنگ را با یک سری از توالی‌های متنقی به گونه‌های مختلف دانالیلا به استناتیس دانالیلا سالیا نازادهای 18S rDNA و دانالیلا وریدیس نشان داد. انتقال CCAP 19/18 و CCAP 19/30 ایزوله مورد مطالعه با اندام دهنده زن مربوط به دانالیلا ترتپولکا و نازادهای ایناگونی به درون گونه دانالیلا سالیا بکسان بود. تجزیه و تحلیل پروتئین این‌گونه نگاری 18S rDNA و آنالیز فیلوپنتیکی آشکار ساخته که دانالیلا ترتپولکا اندازه‌گیری تاکسن به همراه مورد مطالعه است. در مشخصه شوری مطلوب رشد (15-3/3 وزنی-جسمی) و دانالیلا ترتپولکا 7/8 (پیک‌گرم نرم سلول) با دانالیلا گروه شده برای دانالیلا ترتپولکا قابل مقایسه بود. نشان این ایزوله با گونه دانالیلا ترتپولکا از نظر صفات ظاهری نظر اندام دانالیلا، و بدون عملیات به دانالیلا انکارکار یابی شد. در مجموع با درنظر گرفتن بررسی مولکولی و مورفو-فیزیولوژیکی، ایزوله مورد مطالعه به گونه ترتپولکا نسبت داده شد و به عنوان Dunaliella tertiolecta ABRIINW-G3 نام‌گذاری گردید.