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The use of 18S ribosomal DNA, ITS and *rbcL* molecular markers to study the genus *Dunaliella* (Dunaliellaceae) in Iranian samples: A phylogenetic approach

by

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Abstract

The microalga *Dunaliella* has been the focus of attention over recent decades owing to its high biotechnological potential for the production of β-carotene, biofuels and even as a good expression system for the production of recombinant proteins. Different species of this genus have unique features, biological characteristics and biotechnological potential. Therefore, it is necessary to have a clear and reliable taxonomic method to identify different species of *Dunaliella*. Although several taxonomic systems are available for *Dunaliella* based on morphological, physiological and molecular features, none of these methods are reliable enough and some controversies exist over different classification systems. In the current study, molecular techniques and bioinformatics tools have been used to re-assess the phylogenetic position of *Dunaliella* species based on 18S ribosomal DNA (18S rDNA), ITS and *rbcL* regions. The overall findings based on these markers provide a new and more reliable tool for phylogenetic analysis of *Dunaliella* species/strains.

Key words: *Dunaliella*, ITS, microalgae, phylogeny, *rbcL*, 18S rDNA, classification

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Introduction

The genus *Dunaliella* (Dunaliellaceae, Dunaliellales) encompasses bi-flagellate and cell wall-less microalgae that exists in hypersaline environments (Massyuk 1973). Species of this genus are known as the only photosynthetic eukaryotes that can grow in a wide range of salt concentrations, varying from 0.05 to 5.0 M NaCl (Garcia et al. 2007). This microalga was first described by Teodoresco in 1905 (Oren 2005), which was followed by a number of studies aimed at classifying the *Dunaliella* species. Part of the importance of the *Dunaliella* taxon results from its high biological and biotechnological potential in the production of antioxidant pharmaceutical pigments (Barzegari et al. 2010). It also raises hopes for the production of the second and third generation of biofuels. Another relevant application of *Dunaliella* is the production of recombinant proteins, which is to be developed in the future (Dehghani et al. 2018; Dehghani et al. 2017). *Dunaliella* species and strains have many different characteristics such as varying growth rates, sizes, morphological features as well as various requirements regarding the medium, in addition to varied potential. With such parameters, much attention has been paid to developing more robust and reliable approaches to identify different species of *Dunaliella* (Borowitzka & Siva 2007). Until recently, several attempts have been made to encourage novel taxonomic methods to identify *Dunaliella* species. One of the first taxonomic systems for *Dunaliella* species was based on morphological and physiological characteristics (Massyuk 1973). Different *Dunaliella* species were cultured under a wide range of salinity and other cultivation factors to study the variability of morphological and physiological features (Massyuk 1973). However, the use of morphological and physiological characteristics is not an effective tool to unambiguously identify all species of the genus *Dunaliella* (Borowitzka & Siva 2007).

Another well-known taxonomic system of *Dunaliella* was described based on morphological and biochemical criteria. The taxonomy of *Dunaliella* species was revised by Borowitzka based on cell length/width, optimum salinity, stigma condition, flagella length, the existence and type of refractile granules, the type of symmetry, cell color, the maximum total carotenoid content, the type of carotenoid and the formation of aplanospores (Borowitzka & Siva 2007). However, it is known that morphological and even physiological features of *Dunaliella* species vary greatly depending on the developmental stage and culture conditions such as nutrient availability, light intensity and temperature fluctuations (Gomez et al. 1999; Markovits et al. 1993; Riisgard et al. 1980). For instance, Ben-Amotz reported that *D. salina* lacks canthaxanthin (a type of carotenoid) in the mature form of the species (Ben-Amotz et al. 1982). However, further research showed that canthaxanthin is a major carotenoid in aplanospores of the species (Borowitzka & Siva 2007). Apparently, ultra-structural studies cannot help to discriminate *Dunaliella* species (Parra et al. 1990). Therefore, new approaches using molecular biological methods are being developed to classify algae, including *Dunaliella* (Olmos et al. 2009).

Today, different molecular markers such as the 18S rDNA gene, the Internal Transcribed Spacer (ITS1 and especially ITS2), the nuclear-encoded SSU rRNA and chloroplast genomes are frequently used in the taxonomy of green microalgae (Coleman et al. 1994; Mai & Coleman 1997; Olmos et al. 2000; Proschold et al. 2001; Lemieux et al. 2015). Gonzalez et al. (2001) for the first time compared the PCR-RFLP patterns of the ITS region in *Dunaliella* species and claimed that ITS is a quick and reliable genetic marker for the discrimination of *Dunaliella* species (Ben-Amotz et al. 2009). Furthermore, the authors studied the ITS diversity in *D. salina*, *D. bardawil*, *D. tertiolecta*, *D. parva*, *D. viridis*, *D. lateralis* and *D. peircei* and isolated them by cluster analysis (Gonzalez et al. 1998). The conserved and variable regions of the 18S rDNA gene were considered as another critical marker for the identification and classification of eukaryotic organisms (Olsen et al. 1986). Surprisingly, studies of *D. parva* and *D. salina* showed that the 18S rDNA gene in these genera contains intron(s), belonging to group I (Wilcox et al. 1992). A set of conserved and specific oligonucleotide primers were designed and successfully used to identify *D. salina*, *D. parva* and *D. bardawil* (Olmos et al. 2009). Further, the *rbcL* gene (encoding the large subunit of RuBisCo) was also used as an adjunct marker for the classification of various plants and microalgae (Fredericg and Ramírez 1996; Freshwater et al. 1994). Remarkably, the *rbcL* marker has not been widely used for the classification of *Dunaliella* species, while this marker can be used to solve many ambiguities in terms of the *Dunaliella* taxonomy and systematics.

In the present study, the molecular identification of *Dunaliella* species from two different saline lakes of Iran was performed based on three key markers, including 18S rDNA, ITS, and *rbcL* markers. These molecular markers represent a newer and more reliable approach, based on which the phylogenetic positions of the *Dunalialla* species and related strains were analyzed and re-evaluated.

Materials and methods

Microalgae isolation

Water samples were collected from Maharlou (29.47°N; 52.77°E) and Bakhtegan (29.35°N; 53.89°E) salt lakes (Fars province) in Iran, then cultured in 500 ml Erlenmeyer flasks containing Walne's medium (Raja et al. 2004). After two weeks, 10 µl from the content of each flask was spread on modified Johnson's solid medium (Johnson et al. 1968). These plates were placed under continuous illumination by white fluorescent lamps for 20 days. Consequently, based on morphological characteristics (the size of algae and position of stigma), single colonies of *Dunaliella* species were picked and transferred into 50 ml Erlenmeyer flasks containing 1 M of modified Johnson's medium and kept at a temperature of 26°C, 16:8 (Light:Dark) photoperiod and 80 µmol photon m^{-2} s^{-1} irradiance.

The major parts of two important saline lakes in Iran (Maharlou & Bakhtegan) have dried up due to a significant decrease in rainfall and changes in ecological conditions. It is therefore necessary to identify and protect valuable microorganisms especially the microalgae in the lakes. Based on the *Dunaliella* identification key (Borowitzka & Siva 2007; Massyuk 1973), using cell color, flagella length and stigma condition, two *Dunaliella* were identified as *Dunaliella salina* isolate BAK (Bakhtegan) and *Dunaliella pseudosalina* isolate MAH (Maharlou). *Dunaliella salina* isolate BAK has green (1M NaCl) to red color (5M NaCl concentration), two flagella equal to cell length and diffuse stigma (Fig. 1a, b). *Dunaliella pseudosalina* isolate MAH has green (at 1M NaCl) to orange color (at 5M NaCl), two cylindrical long flagella and a distinct stigma located on the left side of the body (Fig. 1c, d).

Amplification of 18S rDNA, ITS, and *rbcL*

Genomic DNA extraction was performed on the exponential phase using the CTAB based method (Hejazi et al. 2010). To amplify the 18S rDNA associated with ITS as well as *rbcL* genes, the following primer pairs were respectively used: FP: 5′-TAGTCATATGCTTGTCTCAAAG-3′, RP: 5′-CTATAGACTACAATTCTCCAAAG-3′ and FP: 5′-GCTGCTAATTCAGGAGACCA-3′, RP: 5′-GGTTCCACAAACTGAAACGA-3′. PCR reactions were performed in 25 µl volumes, containing 20 ng of the genomic DNA, 50 ng of each primer, a master mix (Ampliqon company, *Odense*, Denmark) and deionized water. Gene amplification was achieved by a Peqlab thermal cycler (Model: Primus 96 advanced, Wilmington, USA) as follows: 4 min at 94°C for the initial denaturation time, 32 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 2 min, with the final extension step at 72°C for 10 min. Finally, the PCR products were electrophoresed using 1% agarose gel.

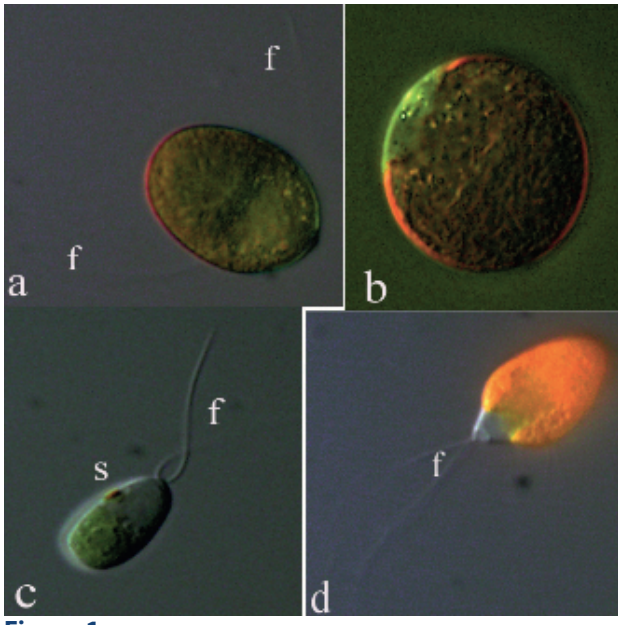

Figure 1

Morphological characteristics of microalgae. *D. salina* isolate BAK (from Lake Bakhtegan) at 1M NaCl (a) and at 5M NaCl (b); and *D. pseudosalina* isolate MAH (from Lake Maharlu) at 1M NaCl (c) and at 5M NaCl (d). f – flagella and s – stigma

The Restriction Fragment Length Polymorphism (RFLP) analysis for the amplified 18S rDNA RFLP analysis was carried out at a total volume of 20 µl using 10 µl of the amplified 18S rDNA gene (0.5 µg of DNA), 0.5 µl of the Hhal restriction enzyme (Thermo Fisher Scientific, Waltham, USA), 2 µl of the Tango buffer (10X) and 7.5 µl of deionized water incubated at 37°C for 5 h. Consequently, restriction fragments were electrophoresed on 1.2% agarose gel.

Results

Molecular techniques and bioinformatics tools were used to identify the samples and re-evaluate the phylogenetic position of *Dunaliella* species based on the 18S rDNA, ITS and *rbcL* regions. Specific primers were designed to amplify both the 18S rDNA gene and the ITS region. Amplification results showed an approximately 2500 bp band on the electrophoresis gel (Fig. 2a). Digesting the PCR products with the Hhal

restriction enzyme showed different RFLP patterns for the samples (Fig. 2b). Sequencing and blasting against the NCBI database showed high similarity with the sequences of *D. salina* and *D. pseudosalina*.

Moreover, the results of *rbcL* amplification in the *Dunaliella* species revealed the same size (~900 bp; Fig. 2c) with different RFLP patterns on the electrophoresis gel. Subsequently, these PCR products were sequenced and deposited in the NCBI database and based on the blasting results, the strains were named as *D. salina* isolate BAK and *D. pseudosalina* isolate MAH.

value (lnL) and the number of parameters (including branch lengths), the best models were obtained for the phylogenetic analyses. Furthermore, the sequences of 18S rDNA, ITS 1, ITS 2 and *rbcL* of *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Asteromonas gracilis* isolate BA were designated as an outgroup (Tables 1, 2, 3).

Phylogeny based on 18S rDNA

For this purpose, the data were analyzed using the Kimura 2-parameter model and the discrete Gamma

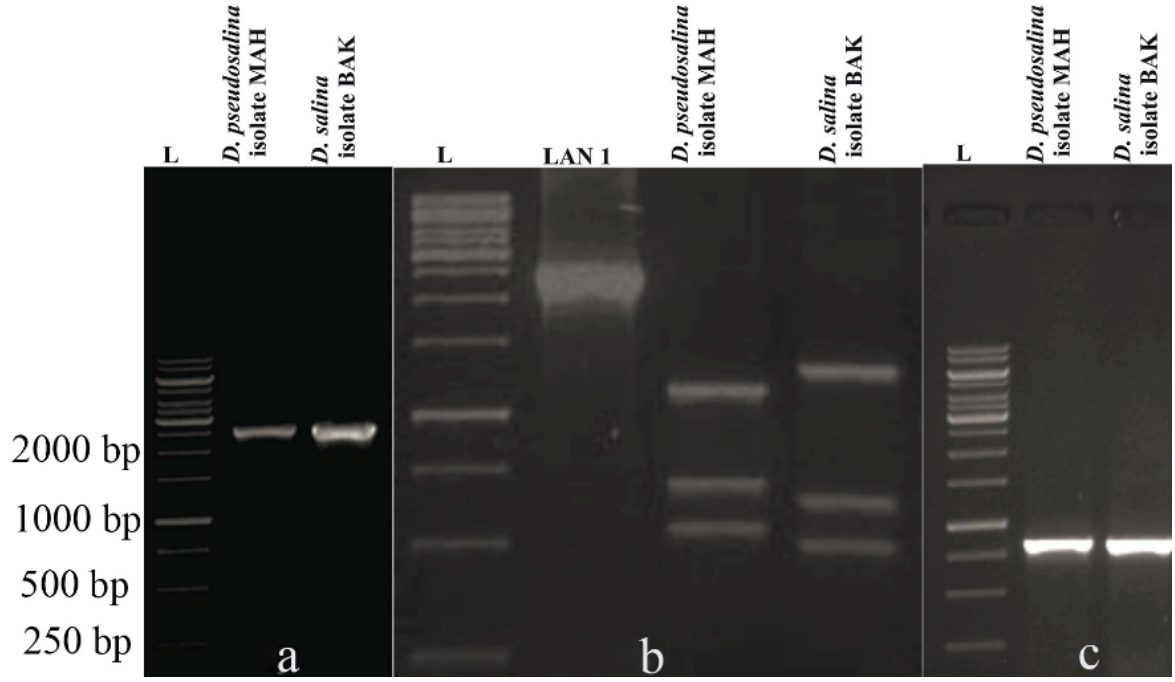

Figure 2

PCR products of 18S rDNA and ITS (~2500 bp) in *D. pseudosalina* isolate MAH and *D. salina* isolate BAK (a). RFLP pattern of PCR products presented in gel after digestion by HhaI (restriction enzyme) (b). PCR products of *rbcL* gene (~900 bp) in *D. pseudosalina* isolate MAH and *D. salina* isolate BAK (c). "Lan" 1 indicates the 18S rDNA PCR product of *D. salina* isolate BAK and "L" indicates Ladder

Phylogenetic analyses

The *Dunaliella* species with 18S rDNA, ITS (ITS 1 + ITS 2) and *rbcL* sequences registered in the NCBI database is associated with two isolated *Dunaliella* strains that were phylogenetically analyzed by MEGA software version X. Consequently, phylogenetic analyses were performed employing the maximum likelihood (ML) by MEGA software version X (Kumar et al. 2008). On the basis of the Bayesian Information Criterion (BIC) scores, the Akaike Information Criterion, corrected (AICc) values, the Maximum Likelihood distribution based on the numbers of BIC (21263.41) and AICc (20871.44) values. Our ML analysis of the 18S rDNA data supports a big split between *D. lateralis* strain Nepal and *Dunaliella* sp. Atacama with all other members of *Dunaliella*. The results suggest the closer relationship of the species to *C. reinhardtii*, *C. vulgaris*, and *A. gracilis*. In fact, these species are more divergent than other species of the genus *Dunaliella*. The strains KMMCC 1346 and UTEX LB 2538 of *D. bardawil* were classified together in the top of the phylogenetic tree in clade A. Moreover, *D. salina* isolate BAK was grouped with *D. pseudosalina* isolate MAH through

Table 1

The 18S rDNA sequences of *Dunaliella* spp. from the NCBI database

Dunaliella spp.	Gene size (bp)	Accession number	Geographic origin
D. bardawil strain KMMCC 1346	2054	JQ315779.1	Republic of Korea
D. bardawil UTEX LB 2538	2088	DQ009777.1	USA
D. bioculata UTEX LB 199	1687	DQ009761.1	USA
D. parva	2585	M62998.1	Unknown
D. peircei strain UTEX LB 2192	2065	DQ009778.1	USA
D. primolecta UTEX 1000	1620	KJ018734.1	USA
D. salina strain KMMCC 1428	1647	JQ315781.1	Korea
Dunaliella sp. ABRIINW M1/2	2120	EU678868.1	Iran
D. salina UTEX LB 200	2065	DQ009779.1	USA
D. salina strain KU07	2069	KF825551.1	Thailand
D. salina strain KU11	2067	KF825550.1	Thailand
D. salina strain KU13	2068	KF825552.1	Thailand
D. salina isolate BAK	1784	KU641617	Iran
Dunaliella sp. SAS11133	1722	KF054056.1	China
D. pseudosalina isolate MAH	1735	KU641615	Iran
D. salina isolate BAK	1784	KU641617.1	Iran
Asteromonas gracilis isolate BA	1687	KU351659.1	Iran
D. viridis strain CONC002	2494	DQ009776.1	USA
D. tertiolecta CCMP 364	1620	KJ018735.1	USA
D. tertiolecta UTEX 999	1620	KJ018733.1	USA
Chlamydomonas reinhardtii	1641	AB701555	Japan
D. lateralis strain Nepal	1692	DQ009762.1	USA
Chlorella vulgaris	1770	KJ756813	UK
D. polymorpha	2991	KJ756825.1	UK

Table 2

ITS1 and ITS 2 sequences of *Dunaliella* spp. from the NCBI database

Dunaliella spp.	ITS1 size	ITS2 size	Accession number	Geographic origin
D. bardawil strain KMMCC 1346	214	230	JQ315779.1	Republic of Korea
D. bardawil UTEX 2538	210	232	DQ377085.1	USA
D. biocolata strain UTEX 199	209	328	DQ377086.1	USA
D. parva	213	226	DQ116746	China
D. peircei strain UTEX 2192	210	226	AF313442.1	Chile
D. primolecta UTEX 1000	210	328	DQ377092.1	USA
D. acidophila strain CCAP 19/35	213	232	HM060646.1* HM060645.1●	Spain
D. salina strain KMMCC 1428	214	227	JQ315781.1	Republic of Korea
Dunaliella sp. ABRIINW M1/2	220	225	EU927373.1	Iran
D. Salina UTEX 200	209	227	DQ313197.1	Chile
D. salina strain KU07	159	229	KF825555.2	Thailand
D. salina strain KU11	159	229	KF825549.1	Thailand
D. salina strain KU13	159	229	KF825547.1	Thailand
D. salina isolate BAK	216	233	KU641617	Iran
Dunaliella sp. SAS11133	204	230	KF054058.1	China
D. pseudosalina isolate MAH	212	225	KU641615	Iran
Asteromonas gracilis isolate BA	214	230	KU351659.1	Iran
D. viridis strain CONC002	217	228	DQ377098.1	USA
D. tertiolecta CCMP 364	210	228	DQ377097.1	USA
D. tertiolecta UTEX 999	210	226	AF313434.1* AF313435.1●	Chile
Chlamydomonas reinhardtii	205	244	U66954	Japan
D. lateralis strain Nepal	212	201	DQ377089.1	USA
Chlorella vulgaris	291	404	KJ756813	UK
D. polymorpha	213	227	KJ756825	UK

* indicates ITS1 and ● indicate ITS2 accession number

Table 3

The *rbcL* sequences of *Dunaliella* spp. from the NCBI database

Dunaliella spp.	rbcL size	Accession number	Geographic origin
Dunaliella bardawil strain KMMCC 1346	798	JQ315489.1	Republic of Korea
Dunaliella bardawil UTEX 2538	1038	DQ313194.1	USA
D. biocolata strain UTEX 199	1038	DQ313195.1	USA
D. parva	1040	DQ173091.1	Chila
D. peircei strain UTEX LB 2192	869	DQ313196.1	USA
D. primolecta UTEX 1000	1038	DQ313198.1	USA
D. acidophila strain CCAP 19/35	667	HQ142901.1	Spain
D. salina strain KMMCC 1428	894	JQ315491.1	Korea
Dunaliella sp. ABRIINW M1/2	1320	KC149893.1	Iran
D. salina UTEX 200	869	DQ313197.1	USA
D. salina strain KU07	427	KF825555.2	Thailand
D. salina strain KU11	613	KF825554.1	Thailand
D. salina strain KU13	632	KF825553.1	Thailand
D. salina isolate BAK	789	KU682279	Iran
Dunaliella sp. SAS11133	717	KF054057.1	China
D. pseudosalina isolate MAH	799	KU641616	Iran
D. viridis strain CONC002	1038	DQ313206.1	USA
D. tertiolecta CCMP 364	1038	DQ313204.1	USA
D. tertiolecta UTEX 999	1038	DQ313203.1	USA
Chlamydomonas reinhardtii	1128	AB511846	Japan
Chlorella vulgaris	1428	AB260909	Japan
Asteromonas gracilis	469	JN033249.1	Chile

high bootstrap values (90%). The KMMCC 1428 strain of *D. salina* is also more closely related to clade B. The KU07, KU11, and KU13 strains of *D. salina* were located together in clade C (100% bootstrap values). In addition, *D. parva* and *D. polymorpha* strain CCAP 19/14 were clustered together in clade D (92% bootstrap values; Fig. 3).

ITS based phylogeny

The ITS data were analyzed employing the Kimura 2-parameter model and the discrete Gamma distribution (K2 + G) based on the numbers of BIC (5759.90) and AICc (5421.40) values. Our ITS phylogenetic analyses showed that this marker could not be used solely for the classification of *Dunaliella* species. However, inconsistent with 18S rDNA, this approach designated *D. lateralis* strain Nepal as an outgroup in the phylogenetic tree, showing a great divergence with the other *Dunaliella* species (Fig. 4). The strains CCMP 364 and UTEX 999 of *D. tertiolecta*, *D. bioculata* strain UTEX 199, and *D. parva* are clustered together in clade A. The ITS data strongly support *D. bardawil* strain UTEX 2538 and *D. primolecta* strain UTEX as sister strains (clade B). Moreover, *Dunaliella* sp. SAS11133 and *D. salina* isolate BAK are grouped together with high support values (98%). Interestingly, *D. viridis* strain CONC002 and *D. polymorpha* strain

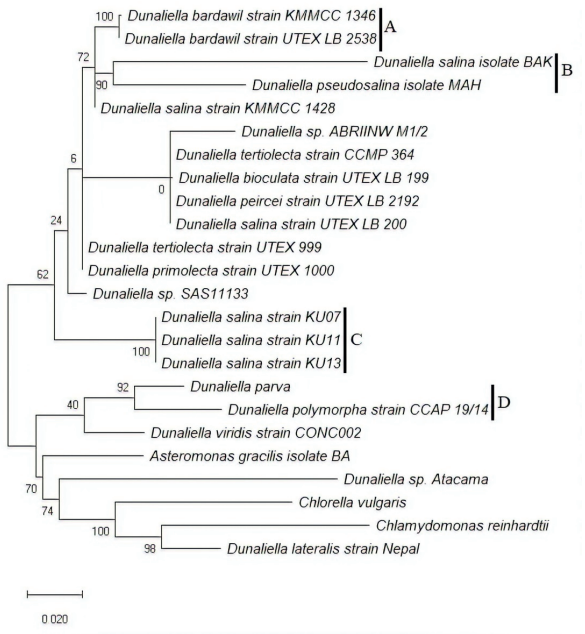

Figure 3

The 18S rDNA based phylogenetic tree. The tree was obtained by the ML method (K2 + G model) with 500 bootstrap replications. The capital letters (A–D) show the clades.

CCAP 19/14 are also clustered together with 100% support values. According to reports based on the 18S rDNA, the KU07, KU11, and KU13, strains of *D. salina* were grouped together (100% bootstrap values) and they are more closely related to *Dunaliella* ABRIINW M1/2 (Fig. 4).

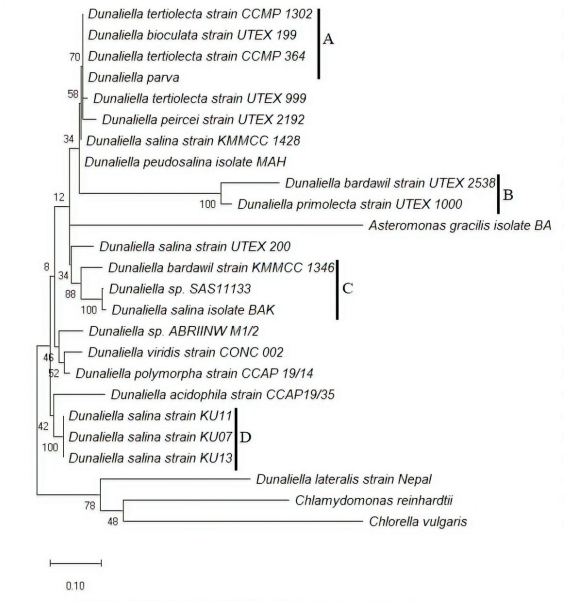

Figure 4

The ITS based phylogenetic tree. The tree was obtained by the ML method (K2 + G model) with 500 bootstrap replications. The capital letters (A–D) show the clades.

Phylogenetic analysis based on *rbcL*

The *rbcL* data were analyzed using the Tamura 3-parameter and the discrete Gamma distribution (T92 + G) based on the numbers of BIC (3456.53) and AICc (3145.85) values.

Our phylogenetic analyses based on the *rbcL* marker using the ML method showed that *D. parva*, *D. pesudosalina* isolate MAH and *D. tertiolecta* strain CCMP 346 are more closely related. Moreover, *D. salina* UTEX 200 and *D. peircei* UTEX LB 2192 are located in the same clade (86% bootstrap values), where *Dunaliella* sp. SAS11133 is closer to the members of this clade (100% bootstrap values). In addition, the *rbcL* data revealed that the UTEX 2538 and KMMCC 1346 strains of *D. bardawil*, *Dunaliella* ABRIINW M1/2, and *D. salina* isolate BAK are evolutionarily related. The KU07, KU11 and KU13 strains of *D. salina* are located together in the same clade, and accordingly they are sister strains. Surprisingly, based on the *rbcL* data, *D. acidophila* is more closely related to the members of clade D. Moreover, based on the *rbcL* data, *D. viridis* strain CONC 002 is highly divergent from the other *Dunaliella* strains (Fig. 5). To date, the *rbcL* sequence of *D. lateralis* strain Nepal has not been reported, and thus its taxonomic position remains unclear.

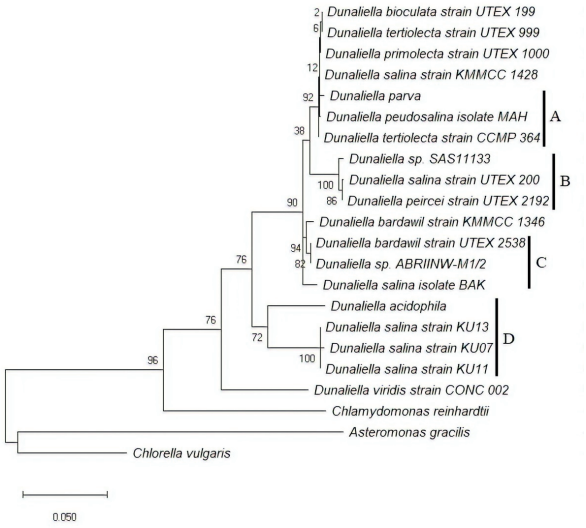

Figure 5

The *rbcL* based phylogenetic tree. The tree was obtained by the ML method (T92 + G model) with 500 bootstrap replications. The capital letters (A–D) indicate the clades.

Combined phylogenetic analysis based on 18S rDNA, ITS, and *rbcL*

The data were analyzed employing Tamura-Nei and the discrete Gamma distribution (TN93 + G) based on the numbers of BIC (22 520.49) and AICc (22 107.66) values. The ML phylogenetic analysis based on the combined 18S rDNA, ITS and *rbcL* sequences showed that *D. lateralis* strain Nepal is highly divergent compared to other *Dunaliella* species. Thus, in the presence of three different genera of microalgae (i.e. *C. reinhardtii*, *C. vulgaris*, and *A. gracilis*), *D. lateralis* strain Nepal is placed as an outgroup. Moreover, *D. bioculata* strain UTEX 199, *D. parva* and *D. salina* strain KMMCC 1428 are evolutionary related (clade A). *D. pesudosalina* isolate MAH and *D. salina* isolate BAK as well as *D. peircei* UTEX LB 2192 and *D. salina* UTEX 200 are clustered together in clade B and C, respectively. Further, the CCMP 364 and UTEX 999 strains of *D. tertiolecta* and *D. primolecta* strain UTEX 1000 are clustered together with high bootstrap values (clade D). In addition, KU07, KU11 and KU13 strains of *D. salina*, *Dunaliella* ABRIINW M1/2, and *Dunaliella* sp. SAS 11133 are grouped together with 100% support values (Fig. 6).

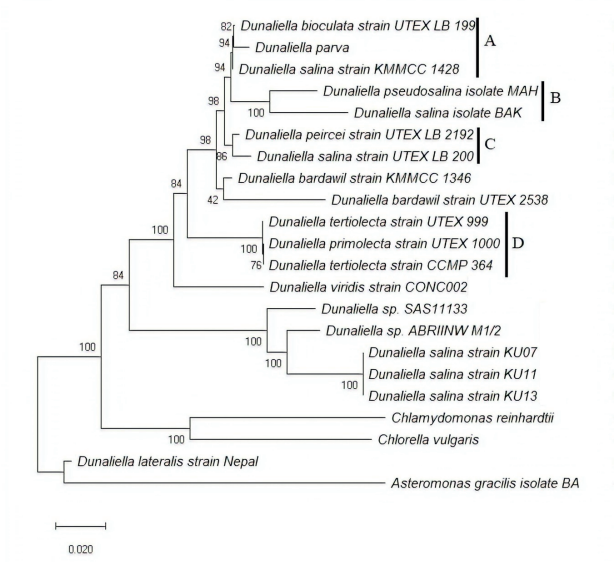

Figure 6

Combined 18S rDNA, ITS and *rbcL* based phylogenetic tree. The tree was drawn using the ML method (TN93 + G model) with 500 bootstrap replications. The capital letters (A–D) indicate the clades.

Discussion

So far, several studies have been performed using different molecular markers for the *Dunaliella* classification. The taxonomy of *Dunaliella* species was revised by the ITS2 secondary structure and compensatory base changes (CBCs). Consequently, *D. primolecta* UTEX 1000 and *D. bioculata* UTEX 199 were renamed as *D. tertiolecta* (Assuncao et al. 2012). This suggestion is consistent with our combined analysis with the18S rDNA, ITS and *rbcL* markers, confirming that *D. primolecta* UTEX 1000 and *D. bioculata* UTEX 199 are clustered with *D. tertiolecta* strains. Furthermore, using the ITS marker, Gonzalez et al. (2001) proposed that *D. percei* UTEX 2192 should be renamed to *D. tertiolecta*. The results obtained in this work also verify these revisions in terms of the ITS data due to the grouping of *D. percei* UTEX 2192 with *D. tertiolecta* strains.

Another ambiguous case is the phylogenetic position of *D. bardawil* UTEX 2538. Based on the morphological features of *D. bardawil* UTEX 2538, it should be considered as *D. salina* (Borowitzka & Huisman 1993). However, all the analyses presented here revealed that *D. bardawil* UTEX 2538 was classified with *D. bardawil* strain KMMCC 1346.

The taxonomic position of *D. salina* UTEX 200 is also very unclear. According to studies of the ITS-RFLP and ITS sequences, this strain is more closely related to *D. pseudosalina* CONC 010 (Gonzalez et al. 2001). For comparison, recent physiological studies showed that *D. salina* UTEX 200 is more similar to *D. viridis* rather than *D. pseudosalina* CONC 010 (Cifuentes et al. 2001). Interestingly, phylogenetic studies using morphological characteristics identified *D. salina* UTEX 200 as a synonym of *D. viridis* (Borowitzka & Siva 2007). In the ITS tree, this strain is closer to *D. bardawil* strain KMMCC 1346, while other data showed that *D. salina* UTEX 200 is closer to *D. peircei* strain UTEX LB 2192.

Dunaliella sp. ABRIINW M1/2 has a different 18S rDNA arrangement with respect to the intron insertion site compared to the other *Dunaliella* strains (Hejazi et al. 2010). As regards the ITS2 secondary structure, it is believed that *Dunaliella* sp. ABRIINW M1/2 should be renamed to *D. viridis* (Assuncao et al. 2012). Consistently, the ITS data showed that *Dunaliella* sp. ABRIINW M1/2 is close to *D. viridis* strain CONC002, while the *rbcL* analysis revealed that the microalga is more closely related to the *D. bardawil* strains.

The 18S rDNA analysis shows that *D. salina* isolate BAK and *D. pseudosalina* isolate MAH are evolutionary close (90% bootstrap values). Based on the previous morphological studies, *D. pseudosalina* is larger than *D. salina* and accumulates a remarkable amount of canthaxanthin (a carotenoid that is not found in *D. salina*). It is believed that *D. pseudosalina* is phylogenetically close to *D. salina* and *D. parva* (Ben-Amotz et al. 1982; Massyuk 1973). Our present data support traditional findings regarding *D. pseudosalina* and *D. salina*. Although *rbcL* data suggest that *D. pesudosalina* isolate MAH is related to *D. parva* and *D. tertiolecta* CCMP 364, *D. salina* isolate BAK is phylogenetically closer to *D. bardawil* strains. Furthermore, our findings confirm that KU07, KU11 and KU13 strains of *D. salina* are similar and are therefore considered as one strain.

Based on the former morphological and molecular data, *D. lateralis* strain Nepal shows a high divergence in relation to the other *Dunaliella* species. The ITS1 and ITS2 phylogenetic analyses showed that freshwater microalga *D. lateralis* strain Nepal is clearly different from the *Dunaliella* strains. Therefore, *D. lateralis* strain Nepal is not considered to be a member of the genus *Dunaliella* (Assuncao et al. 2012; Gonzalez et al. 2001). This concept is also supported by ultra-structural studies and the presence of contractile vacuoles (Borowitzka & Siva 2007; Melkonian & Preisig 1984). Accordingly, the presented analyses strongly support the notion that *D. lateralis* strain Nepal is clearly placed outside the *Dunaliella* phylogenetic tree.

D. acidophila strain CCAP 19/35 (other fresh water *Dunaliella*) is classified within the subgenus *Dunaliella*

based on the ITS and *rbcL* studies (Assunçao et al. 2012), while the morphological data did not confirm this position (Borowitzka & Siva 2007). The ITS analysis revealed that *D. acidophila* strain CCAP 19/35 is clustered within the subgenus *Dunaliella*, nonetheless, it showed greater divergence from the other members of *Dunaliella*. *D. acidophila* strain CCAP 19/35 is genetically similar to *Dunaliella* strains, especially *D. salina* strains from the clade D concerning the *rbcL* data.

In fact, because of the variable morphology as well as the type and content of carotenoids under different environmental conditions, the classical methods cannot provide a reliable and proper approach to the classification of *Dunaliella* species. These characteristics may mislead us in terms of the identification and classification of different *Dunaliella* species. Moreover, 18S rDNA, ITS (ITS1 and ITS2) and *rbcL* genes are newer and more efficient markers for the taxonomy of microalgae. However, as mentioned above, these markers cannot serve as a fully reliable tool for phylogenetic and taxonomic approaches to *Dunaliella* species. We therefore believe that these markers should be used together to assess the phylogenetic position of this genus. Similar to several reports, our findings showed that the *D. lateralis* strain shows high divergence in relation to the other *Dunaliella* strains (Fig. 6).

Based on morphological, physiological and ITS approaches, *D. tertiolecta* and *D. primolecta* are clustered together as the *Tertiolectae* section (Oren 2010). As shown in Figure 6, the technique confirmed that *D. primolecta* strain UTEX 1000 is clustered with the other *D. tertiolecta* strains. In addition, depending on some physiological and biochemical features (e.g. salinity tolerance), it is suggested that *D. peircei* UTEX 2192 and *D. parva* (AC: M62998.1) were incorrectly named (Cifuentes et al. 2001). Accordingly, on the basis of the ITS marker, Gonzalez et al. (2001) stated that *D. parva* is misidentified and this strain should be named as *D. viridis*. However, because of high biodiversity within the ITS sequences of the mentioned strains (Oren 2010), this marker cannot be solely used to classify the strains. The method presented in this study is contrary to a later report, so that *D. parva* is clustered with *D. salina* strain KMMCC 1428 and *D. bioculata* strain UTEX LB 199 (Fig. 6). Similarly, based on the ITS marker, *D. peircei* UTEX 2192 is more closely related to the section *Tertiolectae* and is considered as *D. tertiolecta* (Gonzalez et al. 2001). The presented method revealed that *D. peircei* UTEX 2192 is more closely related to *D. salina* (Fig. 6). More specifically, due to the lack of a precise description and, more importantly, any other available strain for the *Peirceinae* section, the determination of the phylogenetic position of this strain is problematic (Oren 2010).

According to the morphological variability and physiological traits, *D. bioculata* is identified as a form of *D. viridis* (Massyuk 1973). Further studies proposed that *D. bioculata* UTEX 199 could belong to the *Tertiolectae* section (Oren 2010). In comparison, our approach showed that *D. bioculata* UTEX 199 is closer to *D. parva* (Fig. 6).

Traditionally, the section *Dunaliella* includes three species: *D. parva*, *D. pseudosalina*, and *D. salina* (Oren 2010). However, the ITS based analysis showed that *D. pseudosalina* is closer to *D. viridis* (Gonzalez et al. 2001; Oren 2010). The present study showed that *D. pseudosalina* isolate MAH and *D. salina* isolate BAK are clustered together with high bootstrap values, even though *D. parva* is more divergent from these strains. The clustering of *D. parva* into the *Dunaliella* section appears questionable (Oren 2010). Importantly, due to some morphological characteristics (color changeability) of *D. salina* UTEX LB 200, this strain should not be considered as *D. salina* (Oren 2010), while the presented analysis confirmed the report that *D. salina* UTEX 200 is more closely related to *D. salina*.

Some previous reports suggested that *D. bardawil* should be considered as *D. salina* or its variety (Oren 2010). On the other hand, the method presented in this paper confirms the grouping of *D. bardawil* strain UTEX 2538 from the USA and *D. bardawil* strain KMMCC 1346 from Korea (Fig. 6).

In addition, the present method offers a more reliable system for accurate phylogenetic analysis of the *Dunaliella* genus. Therefore, *Dunaliella* sp. ABRIINW M1/2 is more closely related to *D. viridis* and should be renamed as a variety of *D. viridis*. Strains KU07, 11 and 13 of *D. salina* are certainly one strain of *D. salina*. Further, *Dunaliella* sp. SAS11133 should be considered as *D. viridis*. Furthermore, *D. salina* strain KMMCC 1428 is more closely related to *D. parva* and should also be considered as *D. parva* (Fig. 6). Further attention and research are obviously necessary to shed more light on the phylogeny and taxonomy of *Dunaliella*.

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