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Phylogenetic and morphological investigation of a *Dunaliella* strain isolated from Yuncheng Salt Lake, China

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Abstract: A *Dunaliella* strain was isolated from Yuncheng Salt Lake, Shanxi, China (111.05°E, 35.03°N). Morphological characteristics and molecular data were used to evaluate the relationship of this algal strain to other *Dunaliella* strains. Morphology of the isolated strain observed was close to *Dunaliella salina*. Phylogenetic trees were constructed from *rbcL*, *psaB*, ITS (ITS-1+5.8S rDNA+ITS-2) and 18S rDNA sequence data. Phylogenetic analysis of the four gene sequences revealed that the isolated *Dunaliella* strain is closely related to *D. salina* (Dunal) Teodoresco, *D. peircei* Nicolai et Baas-Becking, *D. tertiolecta* Butcher and more likely closer to *D. salina*. Combined morphological characteristics and phylogenetic analysis, the isolated *Duanliella*—designated here as *D.* sp YC01—should be a *Dunialla salina* strain.

Keywords: *Dunaliella*, Morphology, Phylogeny

1. Introduction

The genus *Dunaliella* comprises a group of unicellular, biflagellated green algae that lack cell walls. Cells are ovoid, spherical, or ellipsoid [1]. Members of this genus are the only eukaryotic, photosynthetic organisms known to grow over an extremely wide range of salt concentrations, varying from 0.5 M NaCl up to saturation (5.5 M) [2, 3]. This salt adaption ability is attributed to the production of intracellular glycerol, which acts as an osmotic compatible solute [4]. *Dunaliella* may thus be a convenient model organism for studying salt adaptation in algae [5]. Under certain conditions, such as high light intensity exposure and nutrient limitation, some *Dunaliella* species can accumulate high β-carotene levels equivalent to 10% of their dry biomass [6]. β-carotene is a major source of vitamin A, necessary for proper retinal function, and affects many tissue types [7]. Humans cannot synthesize necessary carotenoids, however, and must obtain them from their diets. Because *Dunaliella* is an ideal natural source of β-carotene, *Dunaliella* β-carotene production plants are in operation in Asia, North America, and Australia [8].

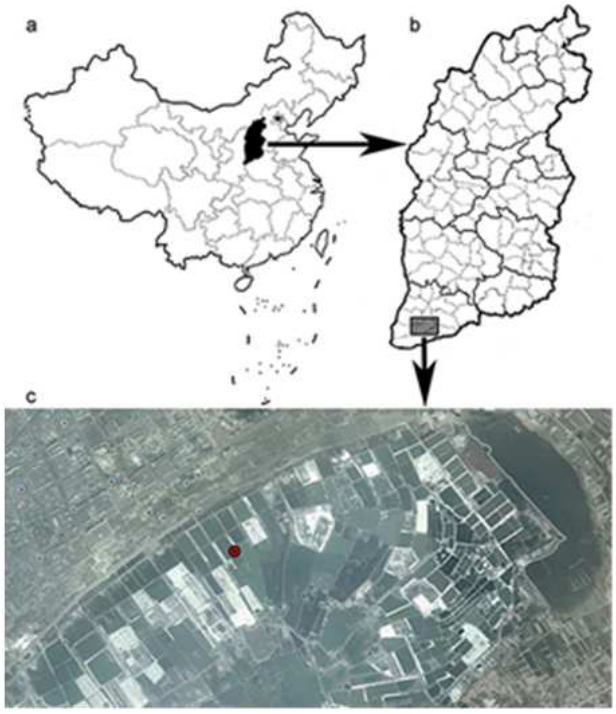
Since the first formal description of *Dunaliella* by Teodoresco [9], species of this genus have been described from a wide variety of habitats [10-12]. Based on a species-level analysis of morphological and structural features combined with physiological and biochemical characteristics, Massyuk [13-15] recognized 29 species, and divided the genus into two subgenera, *Pascheria* and *Dunaliella*. Members of the genus can adapt to extreme environments. For example, *D. acidophila* is able to grow in highly acidic environments (pH 0–1), while a novel subaerial species, *D. atacamensis*, has been described growing on cave spider webs in the Atacama Desert [16]. Because *Dunaliella* species lack cell walls, their morphologies vary under different growing conditions, making characterization difficult. In spite of the excellent work of Massyuk, several strains in culture collections are misidentified, and some species described as separate entities may actually be polymorphic forms of the same taxon [17]. Recent studies have confirmed that molecular methods are useful tools for distinguishing between *Dunaliella* taxa with similar morphologies at inter- and intra-specific levels [18-19]. Combination of morphological data with molecular methods should thus provide a better understanding of *Dunaliella* taxonomy.

In an earlier study, Li and Xie [20] reported the existence of *Dunaliella* in Yuncheng Salt Lake, Shanxi, China. In the study reported here, we isolated and cultured the strain from Yuncheng Salt Lake, and performed a taxonomic and phylogenetic analysis of this micro-alga using both morphological and molecular methods.

2. Material and Methods

2.1. Sample Collection and Isolation

Samples were collected from Yuncheng Salt Lake, Shanxi, China (111.05°E, 35.03°N; Fig. 1). Lake water pH was 7.2–7.6, and its salinity was above 0.1 g ml^{-1}. Isolation and purification of *Dunaliella* cells was performed according to previously described methods [21]. The isolated *Dunaliella* strain was designated as *D.* sp. YC01 in this study.



a. The map of China. The shaded part is Shanxi; b. The map of Shanxi. The shaded part is the location of Yuncheng Salt Lake; c. The map of Yuncheng Salt Lake. The marked part is the sample collection location.

Figure 1. *Sample collection location*

2.2. Culture Conditions

The isolated and purified alga was cultured on medium containing 2.05 M NaCl 17.64 mM, $NaNO_3$, 0.22 mM K_2HPO_4, 0.3 mM $MgSO_4{\cdot}7H_2O$, 0.32 mM $CaCl_2{\cdot}2HO_2$, 0.18 mM Na_2CO_3, 0.02 mM $C_6H_8O_7{\cdot}H_2O$, 10 μM $Fe(NH_4)_3(C_6H_2O_7)$, 46.1 μM H_3BO_3, 9.39 μM $MnCl_2{\cdot}4H_2O$, 0.77μM $ZnSO_4{\cdot}7H_2O$, 1.61 μM $Na_2MoO_4{\cdot}2H_2O$, 0.32μM $CuSO_4{\cdot}5H_2O$, and 0.172 μM $Co(NO_3){\cdot}6H_2O$. *Dunaliella* sp. YC01 was grown in a 250-ml Erlenmeyer flask containing 100 ml medium at 25±1°C, which was manually shaken twice daily and subjected to a 12h:12h light/dark photoperiod provided by cool white fluorescent lamps.

2.3. DNA Methods

After approximately 3 weeks, cultured cells were harvested by centrifugation for 10 min at 5000 x*g*. Genomic DNA was extracted using the CTAB protocol [22]. For amplification of the nuclear 18S rDNA gene region, *Dunaliella*-specific primers MA1 (5'-CGGGATCCGTAGT-CATATGCTTGTCTC-3') and MA2 (5'-CGGAATTCCTT-CTGCAGGTTCACC-3') was used [23]. The chloroplast *rbcL* gene region was amplified using primers 475-497 (5'-CGTGACAAACTAAACAAATATGG-3') and 1181-1160 (5'-AAGATTTCAACTAAAGCTGGCA-3'). The nuclear rDNA ITS region (ITS-1+5.8S rDNA+ITS-2) was amplified using universal primers AB28 (5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3') and TW81 (5'-GGGATCCGTTTCCGTAGGTGAACCTGC-3'). The chloroplast *psaB* gene was amplified using the primers DunpsaBFw22 (5'-ATTTGGGATCCACATTTTGGT-3') and DunapsaBRv753 (5'-TACTGAAGCTAAAGCTAAA-3') [16]. Polymerase chain reactions (PCRs) were performed in a MyCycler thermal cycler (Bio-Rad, California, USA). PCR amplifications of 18S rDNA, *psaB*, and *rbcL* regions were carried out as follows: initial denaturation at 94°C for 5 min, followed either by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min for 18S rDNA, 30 cycles of 93°C for 1 min, 52°C for 1 min, and 72°C for 1 min for *psaB*, or 30 cycles of 94°C for 1 min, 62°C for 50 s, and 72°C for 1 min for *rbcL*, with a final extension of 8 min at 72°C. For the ITS (ITS-1+5.8S rDNA+ITS-2) region, PCRs were performed as described previously [19]. PCR products were analyzed by 1% agarose gel electrophoresis and purified using a Sangon agarose gel DNA extraction kit. The purified products were sent to Sangon Biotech (Shanghai, China) for sequencing.

2.4. Phylogenetic Analyses

Phylogenetic analyses were performed using 18S rDNA, *rbcL*, *psaB*, and ITS sequence data. Multiple alignments incorporating sequences of other *Dunaliella* strains obtained from the National Center for Biotechnology Information (NCBI) nucleotide database (Table 1) were performed using CLUSTALW [24], with manual adjustments as needed. Phylogenetic trees were constructed from the aligned gene sequences using neighbor-joining (NJ), maximum likelihood (ML), and Bayesian (BI) methods. NJ, ML, and BI analyses were performed using MEGA5 [25], PhyML 3.0 [26], and MrBayes version 3.1.2 [27], respectively. For NJ analyses, evolutionary distances were computed using the Kimura 2-parameter method with 1000 bootstrap replicates. The program jModeltest [28] was used to determine the best-fitting models of sequence evolution for ML and BI methods. The model selected for the 18S rDNA data was TrN+I+Γ, with the following parameters used: invariant sites = 0.8532, gamma distribution = 0.6780; base frequencies A = 0.2494, C = 0.2100, G = 0.2755, T = 0.2651; rate matrix A-C = A-T = C-G = G-T = 1, A-G = 4.5699, C-T = 11.4595. For the *rbcL* gene, we used a TPM3uf +I+Γ model, with the following parameters: invariant sites = 0.5750, gamma distribution = 0.5830; base frequencies A = 0.2879, C = 0.2084, G = 0.1878, T = 0.3159; rate matrix A-C = C-G = 0.0970, A-G = C-T = 1.1090, A-T = C-T = 1. Analysis of the *psaB* gene data was carried out under a GTR+Γ model, with gamma distribution = 0.2230 and base frequencies A =

0.2417, C = 0.1841, G = 0.1850, and T = 03892. The model selected for the ITS region was TrNef+Γ, with gamma distribution = 0.4337, rate matrix A-C = A-T = C-G = G-T = 1, A-G = 2.5767, and C-T = 7.0137. *D.* sp. YC01 18S rDNA, *rbcL*, ITS, and *psaB* sequences were deposited in GenBank with accession numbers KF054056, KF054057, KF054058, and KF054059, respectively.

3 Results

3.1. Morphological Observations

Dunaliella sp. YC01 cells were examined and photographed under an optical microscope (Olympus BX-51, Japan). The cells were varied in shape from pyriform to round or ovoid. The cells contained one cup-shaped chloroplast occupying about half of the cell interior, and a single pyrenoid surrounded by several starch bodies. At the flagellar end of cells, a papilla was usually observed in young cells. Flagella were a bit longer than the length of the cell (Fig. 2).

3.2. Phylogenetic Analyses

PCR amplification of *rbcL*, *psaB*, ITS, and 18S rDNA regions of *Dunaliella* sp. YC01 produced 731-, 1,197-, 727-, and 1,721-bp amplicons, respectively. ML, NJ, and BI trees based on each DNA region were constructed from individual aligned datasets comprising sequences of *D.* sp. YC01and other *Dunaliella* strains. For the four genes, topologies recovered based on the BI algorithm are shown in Figs 3-6, respectively, with ML and NJ bootstrap support values indicated.

In the phylogenetic tree generated by BI analysis of *rbcL* sequences (Fig. 3), *D.* sp. YC01 is grouped together with *D. peircei* Nicolai et Baas-Becking strain UTEX 2192 (DQ313196) and *D. salina* (Dunal) Teodoresco strain UTEX 200 (DQ313197) in a well-supported clade (ML bootstrap value/NJ bootstrap value/BI posterior probability of 99.3%/100%/1.00). *D.* sp. YC01 is also shown to be closely related to *D.* sp. MBTD-CMFRI-S086 (JN797810), which is sister to the above clade with ML bootstrap values and BI posterior probabilities of 45.6% and 0.91, respectively. The ML tree was rooted using a published *rbcL* sequence of *Chlamydomonas reinhardtii* P. A. Dangeard as an outgroup [29].

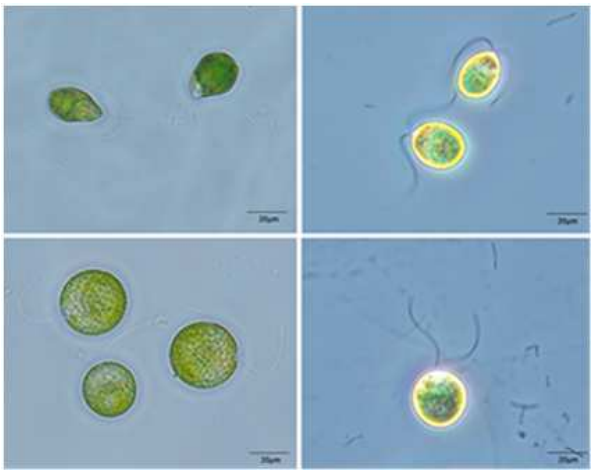


Figure 2. *Morphology of Dunaliella sp. YC01*

In the BI tree obtained using aligned *psaB* gene sequences (Fig. 4), *D.* sp. YC01 is sister to *D. tertiolecta* Butcher (JQ039051). These two strains are placed into a strongly-supported clade (ML/NJ/BI support = 100%/100%/1.00) along with *D. parva* W.Lerche strain UTEX 1983 (AB084375) and *D. salina* (AY820754). The four *Dunaliella* species constitute a monophyletic group in the phylogenetic tree. The tree was rooted using *Chloromonas radiata* (T.R.Deason et H.C.Bold) T.PrÖschold, B.Marin, U.W.SchlÖsser et M.Melkonian [16] as an outgroup.

Using the universal primers AB28 and TW81, the nuclear rDNA ITS region (ITS-1+5.8 rDNA+ITS-2) of *D.* sp. YC01 was amplified; its sequence length, 727 bp, was similar to that of other *Dunaliella* strains in the NCBI database. In the BI tree obtained using aligned ITS gene sequences (Fig. 5). *Dunaliella* sp. YC01 is sister to *D. salina* strain 9802 (EF695045) in a well-supported clade (ML bootstrap value/NJ bootstrap value/BI posterior probability of 98.4%/94%/1.00). This tree was rooted using *Chlamydomonas reinhardtii* [29] as an outgroup.

BI analysis of nuclear 18S rDNA data recovered a phylogenetic tree (Fig. 6) in which *D.* sp. YC01 is sister to *D. salina* (AF506698). These two strains constitute a clade (ML/BI support = 83.4%/0.99) together with *D. salina* strain UTEX LB 1644 (DQ009765). The phylogenetic tree was rooted using a published sequence of *Asteromonas gracilis* Artari strain CCMP 813 [16] as an outgroup. *Asteromonas* is a genus in Asteromonadaceae, the sister family in the Chlamydomonadales to Dunaliellaceae [30-31].

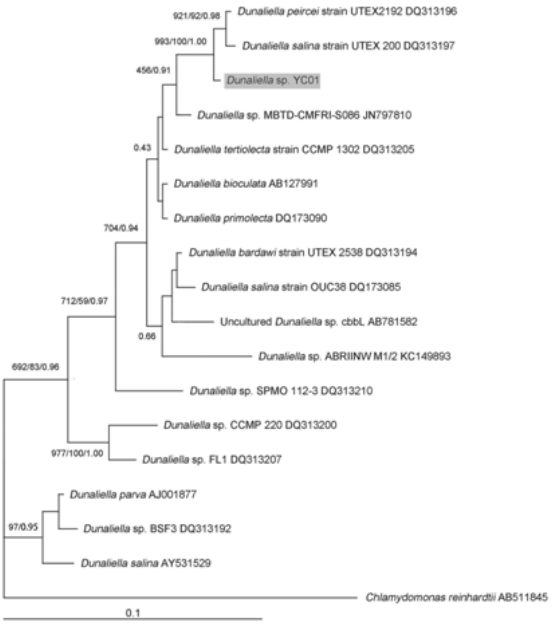


Figure 3. *Phylogenetic tree derived from Bayesian (BI) analysis of aligned rbcL gene sequences of Dunaliella species. Numbers at nodes indicate ML and neighbor-joining (NJ) bootstrap support values derived from 1000 bootstrap replicates, and Bayesian (BI) posterior probabilities (ML/NJ/BI)*

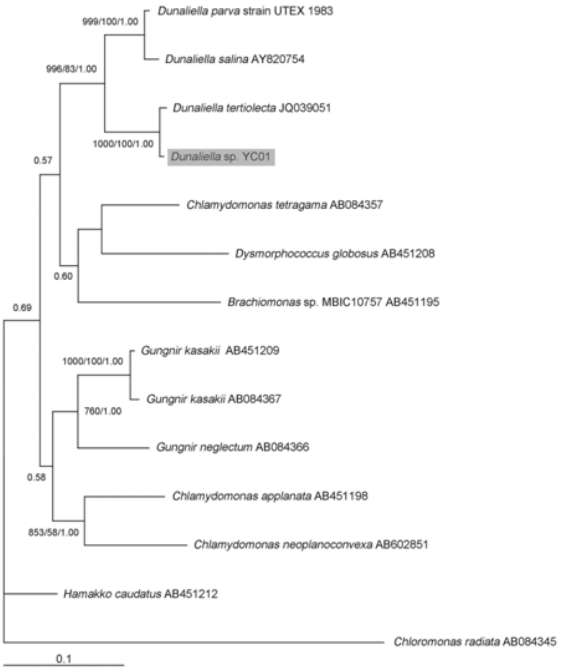


Figure 4. *Phylogenetic tree derived from Bayesian (BI) analysis of aligned psaB gene sequences. Numbers at nodes indicate ML and neighbor-joining (NJ) bootstrap support values derived from 1000 bootstrap replicates, and Bayesian (BI) posterior probabilities (ML/NJ/BI)*

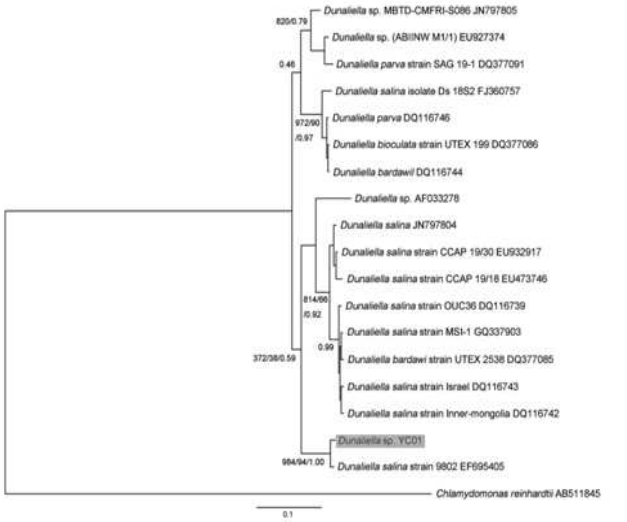


Figure 5. *Phylogenetic tree derived from Bayesian (BI) analysis of aligned ITS sequences of Dunaliella species. Numbers at nodes indicate maximum likelihood (ML) and NJ bootstrap support values derived from 1000 bootstrap replicates, and Bayesian (BI) posterior probabilities (ML/NJ/BI)*

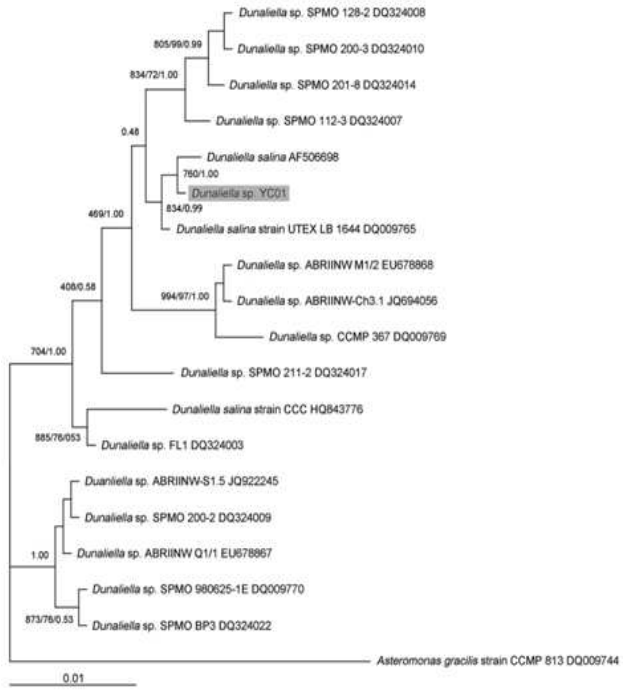


Figure 6. *Phylogenetic tree derived from Bayesian (BI) analysis of aligned 18S rDNA gene sequences of Dunaliella species. Numbers at nodes indicate maximum likelihood (ML) and NJ bootstrap support values derived from 1000 bootstrap replicates, and Bayesian (BI) posterior probabilities (ML/NJ/BI)*

Table 1. *Strains of Dunaliella analyzed in this study, with their geographic origins and GenBank accession numbers*

***Dunaliella* strains**	**18S rDNA**	***rbcL***	***psaB***	**ITS**	**Geographic Origin**
Dunaliella bardawil				DQ116744	
Dunaliella bardawi (UTEX 2538)		DQ313194		DQ377085	salt pond nearBardawil lagoon, North Sinai, Israel
Dunaliella bioculata		AB127991			
Dunaliella bioculata (UTEX 199)				DQ377086	Soviet Union

***Dunaliella* strains**	**18S rDNA**	***rbcL***	***psaB***	**ITS**	**Geographic Origin**
Dunaliella parva		AJ001877			
Dunaliella parva				DQ116746	
Dunaliella parva (UTEX 1983)			AB084375		
Dunaliella parva (SAG 19-1)				DQ377091	LaculSarat, Romania
Dunaliella peircei (UTEX2192)		DQ313196			Salt flat
Dunaliella primolecta		DQ173090			
Dunaliella salina	AF506698				
Dunaliella salina		AY531529	AY820754		
Dunaliella salina				JN797804	Indian
Dunaliella salina (9802)				EF695405	
Dunaliella salina (CCAP 19/18)				EF473746	Hutt Lagoon,Western Australia
Dunaliella salina (CCAP 19/30)				EU932917	salt pond nearBardawil lagoon, North Sinai,Israel
Dunaliella salina (CCC)	HQ843776				Sambar lake, India
Dunaliella salina (Ds 18S2)				FJ360757	
Dunaliella salina (Inner-mongolia)				DQ116742	Inner-Mongolia
Dunaliella salina (Israel)				DQ116743	Israel
Dunaliella salina (MSI-1)				GQ337903	Maharlu salt lake, Shiraz
Dunaliella salina (OUC36)				DQ116739	
Dunaliella salina (OUC38)		DQ173085			
Dunaliella salina (UTEX 200)		DQ313197			Salt flat
Dunaliella salina (UTEX LB 1644)	DQ009765				Baja, Califomia, USA
Dunaliella sp.				AF033278	
Dunaliella sp. (ABRIINW-Ch3.1)	JQ694056				Iran
Dunaliella sp. (ABIINW M1/1)				EU927374	Iran
Dunaliella sp. (ABRIINW M1/2)	EU678868	KC149893			Iran
Dunaliella sp. (ABRIINW Q1/1)	EU678867				Iran
Duanliella sp. (ABRIINW-S1.5)	JQ922245				Iran
Dunaliella sp. (BSF3)		DQ313192			Salt flat
Dunaliella sp. (CCMP 220)		DQ313200			
Dunaliella sp. (CCMP 367)	DQ009769				
Dunaliella sp.(FL1)	DQ324003	DQ313207			Salt flat
Dunaliella sp. (MBTD-CMFRI-S086)		JN797810		JN797805	Indian
Dunaliella sp. (SPMO 112-3)	DQ324007	DQ313210			Salt flat
Dunaliella sp. (SPMO 128-2)	DQ324008				Salt flat
Dunaliella sp. (SPMO 200-2)	DQ324009				Salt flat
Dunaliella sp. (SPMO 200-3)	DQ324010				Salt flat
Dunaliella sp. (SPMO 201-8)	DQ324014				Salt flat
Dunaliella sp. (SPMO 211-2)	DQ324017				Salt flat
Dunaliella sp. (SPMO BP3)	DQ324022				Salt flat
Dunaliella sp. (SPMO 980625-1E)	DQ009770				Salt flat
Dunaliella tertiolecta			JQ039051		
Dunaliella tertiolecta (CCMP 1302)		DQ313205			
Uncultured *Dunaliella* sp. (cbbL)		AB781582			Egyptian

4. Discussion

Morphology of *Dunaliella* sp. YC01 observed was close to *Dunaliella salina*, and with limit of nutrient, the green *D.* sp. YC01 in medium would turn to red, which is consistent to *D. salina*.

Several different molecular marks, primarily 18S rDNA, *rbcL*, ITS, and *psaB*, have previously been used for phylogenetic analysis of *Dunaliella* [23, 16, 3, 19, 29]. Because these molecular marks have different evolutionary rates, they can be used for inter- and intra-specific classification of *Dunaliella*. In our study, phylogenetic analysis of the *rbcL* gene indicated that the closest relatives of *D.* sp.YC01 are *D. peircei* strain UTEX 2192 (DQ313196) and *D. salina* strain UTEX 200 (DQ313197). Blast searching against *psaB* gene sequences in GenBank uncovered only three *Dunaliella* species with high identity to *D.* sp. YC01. Our phylogenetic analysis based on this gene indicated that *D. tertiolecta* (JQ039051) is closely related to *D.* sp. YC01. Phylogenetic analysis of 18S rDNA indicated that the closest relatives of *D.* sp. YC01 are *D. salina* (AF506698) and *D. salina* strain UTEX LB 1644 (DQ009765). Phylogenetic analysis of ITS gene indicated that the closest relative of *D.* sp. YC01 is *D.* salina strain 9802 (EF695405).

Phylogenetic analysis with the four molecular makers indicated the relatives of *D.* sp YC01 are *D. salina*, *D. peircei* and *D. tertiolecta*, and *D. salina* is more likely closer to *D.* sp YC01.

5. Conclusion

Since morphological characteristics of *Dunaliella* sp. YC01 is similar to *D. salina*, and phylogenetic analysis with *rbcL*, *psaB*, ITS and 18S rDNA gene sequences indicated

that the most likely relative of *D.* sp YC01 is *D. salina*. Combined morphological characteristics and phylogenetic analysis, *D.* sp. YC01 should be a *D. salina* strain.

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