Identification of Dunaliella Viridis Using its Markers

[1]Duc Tran*, [2]Clifford Louime
[1]Trung Võ, [3]Mario Giordano
[4]Sixto Portilla, [1]Nguyen Doan
[1]Dat Tran, [1]Truc Mai, [5]Le Bui

[1]School of Biotechnology
International University
Thu Duc Dist., VNU-Vietnam

[2]Florida A and M University
College of Engineering Sciences
Technology and Agriculture
FAMU BioEnergy Group
Tallahassee, FL 32307
USA.

[3]Dipartimento di Scienze Della Vita e Dell'Ambiente (DISVA)
Università Politecnica Delle Marche
Via Brecce Bianche, 60131 Ancona
Italy

[4]Center for Estuarine
Environmental and Coastal Oceans Monitoring
Dowling College, 150 Idle Hour Blvd
Oakdale, New York 11769
[5]Department of Biology
University of Science, VNU
Vietnam.

Abstract

The phylogenetic position and taxonomic status of the green alga Dunaliella viridis was investigated based on internal transcribed spacer (ITS) markers. The alga was isolated from saltern in Vinh Hao, Binh Thuan province, Vietnam. Independent phylogenetic trees of ITS1 and ITS2 sequences revealed that the alga belongs to the clade of Dunaliella viridis. The salinity for optimal growth of the alga was 2M NaCl, which was much lower than the original sampling site (4M NaCl). This tolerance to a wide range of salinity may provide distinct advantages to Dunaliella viridis over its competitors in natural environments. Further physiological and biochemical characteristics of this strain will need to be investigated in order to assess its potential for algal biomass production and other applications such as beta-carotene, carbohydrate, lipid and protein for feed, food, aquaculture and biofuels, including opening new search for other Dunaliella species.

Key Words: Algae, biotechnology, carotene, *Dunaliella*, ITS, phylogenetic tree.

Introduction

Unicellular green algae *Dunaliella* belong to the Chlorophytes (Ginzburg 1987, Pick 1992, Oren 2005). The algae was first described by Dunal in the 1830s (Dunal 1838), but it was not until 1905 that the name *Dunaliella* was given by Teodoresco (Teodoresco 1905). There are currently 23 recognized *Dunaliella* species (Massjuk 1972, Pick 1992, Oren 2005). *Dunaliella salina* TEODORESCO is the type species of the genus, whose vegetative cells are capable of turning red under stress environments such as high irradiance, high salinity, or low nutrient concentrations (Teodoresco 1905&1906, Hamburger 1905, Labbe 1925, Lerche 1937).

*Corresponding Author

Cells of *Dunaliella* are generally ovoid, 4-15 μm wide, and 6-25 μm long, but depending on stages of growth or development and environmental conditions, the cell shape can vary from ovoid, ellipsoidal, cylindrical, pyriform, or fusiform to almost spherical (Teodoresco 1906, Butcher 1959b, Massjuk 1973a&b&c). *Dunaliella* cells are motile with two equally long flagella. The main morphological characteristic of *Dunaliella* is the lack of a rigid polysaccharide wall (Gibbs and Duffus 1976); instead, cells are covered by amorphous mucilaginous layer of variable thickness called a glycocalyx. *Dunaliella* cells contain a cup-shaped chloroplast with a pyrenoid in the center surrounded by starch which is the storage product. The nucleus is located in the colorless anterior portion of the cells (Baas-Becking 1931).

Currently *Dunaliell*a is placed in the order of *Chlamydomonadales*, and the family of *Dunaliellaceae* according to NCBI database (Polle et al. 2009). Because of variation in morphology within a single species under different stages of growth, development and environmental conditions, it is possible that some species were previously misidentified (Borowitzka & Borowitzka 1988, Gonzalez et al 1999, Borowitzka and Siva 2007). According to some more recent phylogenetic studies (Gonzalez et al. 1999, Gonzalez et al. 2001, Gomez & Gonzalez 2004), it is believed that the number of *Dunaliella* species may be less than 23. During the last decade, nuclear rDNA internal transcribed spacers ITS1 and ITS2 have most been used to delineate *Dunaliella* species (Gonzalez et al. 1999, Gonzalez et al. 2001).

Dunaliella can be found on all continents and in oceans, salterns and most hypersaline lakes all over the world. Temperature, salinity and nutrients are limiting factors on the growth and development of *Dunaliella* (Ginzburg 1987). *Dunaliella* were found in the Great Salt Lake (Post 1977), the Dead sea (Oren and Shilo 1982; Oren 2005), and from Antarctic salt lakes to salt lakes in Africa, America, Asia, Australia, and Europe (Ginzburg 1987, Borowitzka & Borowitzka 1988, Lerche 1937). It is therefore hypothesized that strains of *Dunaliella* could exist in Vietnam.

Material and Method

Sample Collection and Isolation

Algal samples were collected from salterns in Vinh Hao, Binh Thuan province, then plated on agar medium according to Uri Pick (1989) with salinity corresponding to the collection site. The medium contained 0.4M Tris-HCl, 5mM KNO3, 5mM MgSO4, 0.3mM CaCl2, 0.2mM KH2PO4, 1.5μM FeCl3 in 6μM EDTA, 0.185mM H3BO3, 7μM MnCl2, 0.8μM ZnCl2, 0.2nM CuCl2, 0.2μM Na2MoO4, 20nM CoCl2, 50mM NaHCO3. Colonies of algae appearing on plate after about two weeks were picked using sterile toothpick and continuously stroke on agar petri plates until axenic alga was obtained.

Molecular Identification

Genomic DNA of *Dunaliella* was isolated using DNeasy plant mini kit following instructions from Qiagen (Cat.No 69104). Isolated DNA was checked by electrophoresis on 1% agarose gel in 1X TAE buffer (50X TAE: 242g Tris-base, 57.1 ml acetic acid, 100ml 0.5M EDTA) and was quantified by spectrophotometer at an OD of 260nm, and frozen at -20^{oC} until being used. Internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2) were respectively PCR amplified using GoTag PCR core system II (Cat. No M7665) from Promega with pairs of primers shown in table 1. The products were checked by electrophoresis on 1% agarose gel in 1X TAE buffer. The products were purified using Wizard SV Gel and PCR clean-up kit from Promega (Cat. No A9281).

Table 1: Primer pairs and sequences were used for PCR amplification

Primers	Sequences
ITS1	Its1F: 5'TCCGTAGGTGAACCTGCGG3' Its1R: 5'GCTGCGTTCTTCATCGATGC3'
ITS2	Its2F: 5'GCATCGATGAAGAACGCAGC3' Its2R: 5'TCCTCCGCTTATTGATATGC3'

Note: F: Forward, R: reverse

The sequenced ITS1, ITS2 were aligned with respective sequences of *Dunaliella* strains obtained from NCBI (http://www.ncbi.nlm.nih.gov/) using Bioedit program version 7.1.3.0 (Hall 1999) (**Table 2, only ITS1 sequences are shown as example**).

Phylogenetic trees were constructed using the Seqboot, Neighbor, and Consense programs in the Phylip package, version 3.66 (Felsenstein 1989). Bootstrap support values were derived from 100 randomized, replicate datasets.

Table 2: ITS1 sequences of Dunaliella strains obtained from NCBI were used for building phylogenetic trees

Names	Accession number	Note
Isolated *Dunaliella viridis*	**KC686614**	**DunaB_Its1**
Chlamydomonas	JX839532.1	chlamy32.1
Chlamydomonas	JX839533.1	chlamy33.1
Chlamydomonas	JX839534.1	chlamy34.1
Dunaliella viridis	AF313418.1	Virid418.1
Dunaliella viridis	AY686682.1	Virid682.1
Dunaliella viridis	AY878699.1	Virid699.1
Dunaliella bardawil	AF313430.1	Barda430.1
Dunaliella salina	AF313422.1	Salin422.1
Dunaliella salina	AF313424.1	Salin424.1
Dunaliella salina	AY545543.1	Salin543.1
Dunaliella salina	HM035323.1	Salin323.1
Dunaliella salina	HM035338.1	Salin338.1
Dunaliella salina	HM035340.1	Salin340.1
Dunaliella salina	HM035336.1	Salin336.1
Dunaliella salina	AY545542.1	Salin542.1
Dunaliella salina	AF313428.1	Salin428.1
Dunaliella salina	HM140783.1	Salin783.1
Dunaliella salina	AF313426.1	Salin426.1
Dunaliella tertiolecta	HM243580.1	Terti580.1
Dunaliella tertiolecta	AF313432.1	Terti432.1
Dunaliella tertiolecta	AF313434.1	Terti434.1
Dunaliella tertiolecta	AY686683.1	Terti683.1
Dunaliella bioculata	HM035325.1	Biocu325.1
Dunaliella bioculata	DQ182330.1	Bio330.1
Dunaliella polymorpha	DQ157050.1	Polym050.1
Dunaliella perceive	AF313442.1	Perci442.1
Dunaliella parva	AF313436.1	Parva436.1
Dunaliella parva	AF313438.1	Parva438.1
Dunaliella minuta	HM035326.1	Minut326.1
Dunaliella quartolecta	DQ157051.1	Quart051.1
Dunaliella primolecta	DQ157052.1	Primo052.1

Note: Chlamydomonas ITS1 sequences were used as outgroup

Salinity Test

The alga was grown in five different salinity (1M, 2M, 3M, 4M, 5M) for over 2 weeks. Optical density was taken every three days using microplate reader (Biotek, Synergy HT) at 750nm (OD_{750}) (cell density was diluted if the OD was above 1.0)

Statistical Analysis

Data was tested by one-way ANOVA analysis using SPSS software version 16.0. All significant levels were set at $p < 0.05$.

Result and Discussion

Isolation and Cell Description

Using a light microscope, various algal isolates with different salinities were observed for *Dunaliella* cells based on morphological descriptions (Teodoresco 1906, Butcher 1959b, Massjuk 1973a&b&c).

There was no indication of the presence of *Dunaliella* cells from all isolates observed in salinities of 1M, 2M, 3M, except one isolate from 4M NaCl sample. Cells of the alga were green, ovoid, 10 µm wide, and 25 µm long. The cell was motile with two equally long flagella in each cell (**Figure 1**). The cell contained a pyrenoid (**d**) surrounded by starch. There was thick mucus (**b**) outside the cell membrane. The alga was tolerant to a wide range of salinity from 1M to 5M, but optimal salinity for growth at 2M significantly at $p<0.05$(**figure 2**), which is in the optimal range for most *Dunaliella* species reported (Polle 2009, Tran et al. unpublished data). Salinity is one of the key elements for marine algal maintenance and cultivation. The defined optimal salinity will be applied for further optimizing growth conditions to obtain high biomass before subjecting the algae to appropriate stress conditions for other secondary metabolite production such as glycerol, lipid, carotene, proteins or carbohydrate in the common two-phase algal cultivation system (Norihiko et al).

Together these morphological and physiological characteristics indicate that the alga probably belonged to the genus *Dunaliella* (Teodoresco 1906, Butcher 1959b, Massjuk 1973a&b&c). In addition, the alga was tolerant to high salinity (4M), which was probably of hypersaline species of *Dunaliella salina* or *Dunaliella viridis* (Polle et al. 2009). To further confirm whether this was *Dunaliella*, and which *Dunaliella* species it was, it was necessary to use molecular markers of ITS1, ITS2 to delineate (Gonzalez et al. 1999, Gonzalez et al. 2001).

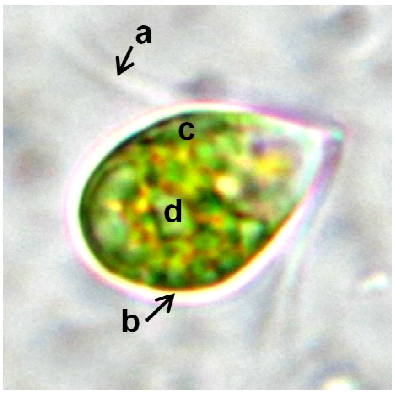

Figure 1: Photo of Dunaliella viridis cell grown in 4M NaCl (a. Flagella, b. Mucilaginous layer, c. Chloroplast, d. Pyrenoid)

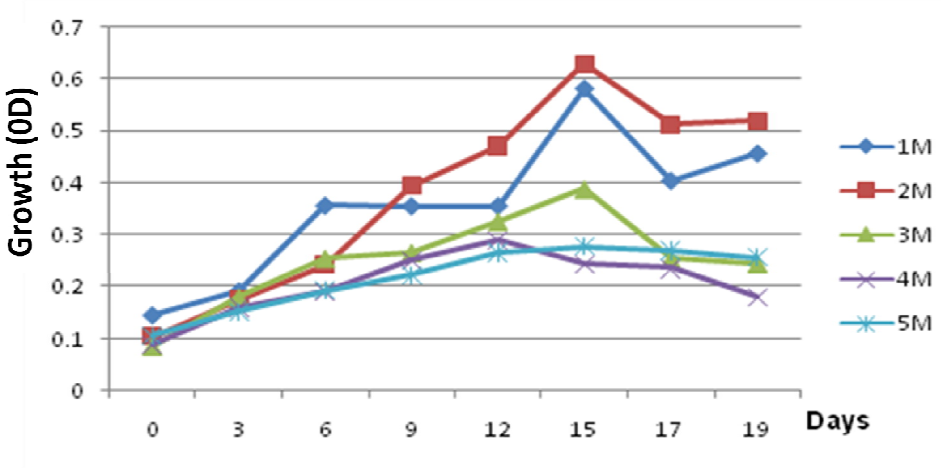

Figure 2: Growth of the isolated Dunaliella viridis in five different salt concentrations (from 1M to 5M). The optimal growth was obtained at the salinity of 2M.

Molecular Identification

The ITS1 and ITS2 sequences of the isolated *Dunaliella* were sequenced and deposited in NCBI (Acc.#: KC686614, KC686615 respectively, will be released soon after being processed). The phylogenetic tree was constructed using ITS1, ITS2, sequences (only ITS1 is shown) (**Figure 3**). Three main clades were clearly separated.

Clade A showed the isolated *Dunaliella* in the group of *Dunaliella viridis* with high boottrap value (92%). Clade B contained all strains of *Dunaliella salina* and *Dunaliella bardawil* which was believed to be a strain of *Dunaliella salina (*Polle et al. 2009*)*. Other *Dunaliella* species (*D. parva, D. percei, D. polymorpha, D. bioculata, D. primolecta*) were all together in clade C, which could be the same species as they were misnamed or misidentified (Borowitzka & Borowitzka 1988, Gonzalez et al 1999, Borowitzka and Siva 2007). Sequences of *Chlamydomonas* obtained from NCBI were used as the out group which formed a separate clade D. Similarly, the phylogenetic trees of ITS2 sequences (data not shown) were in agreement with the topography of the phylogenetic tree for ITS1 sequences. Together, data based on morphology, molecular markers and physiological characteristics (tolerant to a wide range of salinity) (**Figure 2**), this indicates that the alga belongs to *Dunaliella viridis*.

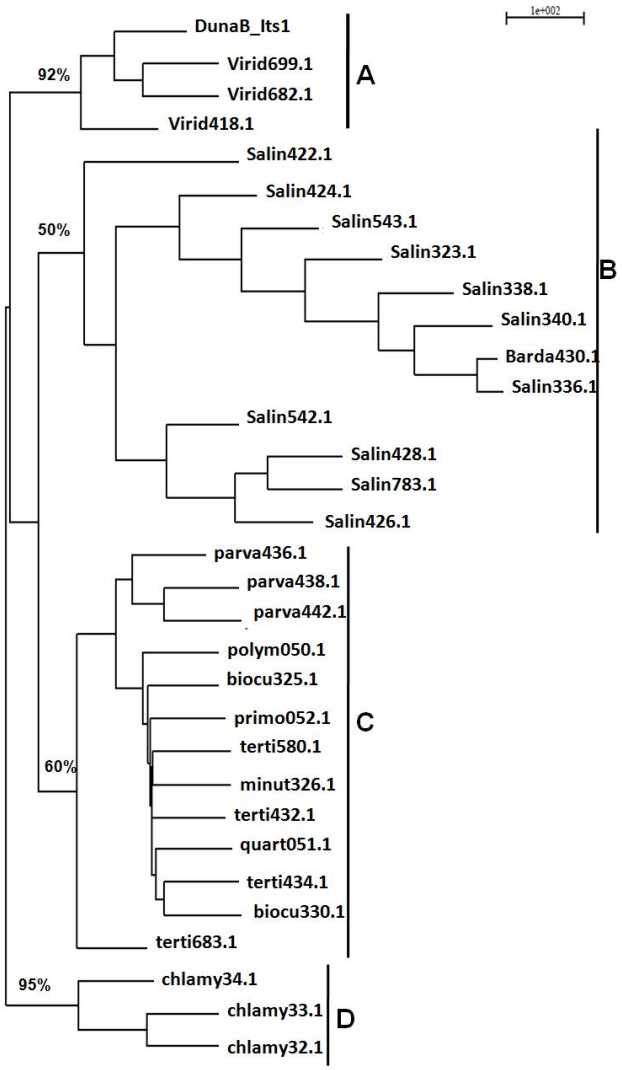

Figure 3: Phylogenetic tree of ITS1 sequences of the isolated Dunaliella viridis (DunaB_Its1) and other Dunaliella species. Chlamydomonas ITS1 sequences were used as the outgroup. (Table 2)

Conclusion

Fossil hydrocarbons have been our main energy sources for centuries. And its use is projected to increase in order to meet the demands of a constantly increasing global population and expanding economy. This will ultimately lead to an unprecedented competition for limited resources. In addition, resulting climate change now seems to negatively impact all segments of the world population. All these factors are presently driving the development of renewable energy sources.

Amongst the proposed alternatives, algal biofuels seem to represent a very attractive and economically viable one, although several challenges must be addressed in order for this technology to become competitive to fossil fuels. Some of these challenges include strain identification and improvement and the production of co-products. In this article, we attempted address the first challenge by identifying *Dunaliella viridis* for the first time in Vietnam based on morphology, physiology and molecular markers. *Dunaliella viridis* has been found to have an optimum growth salinity not previously known. This defined optimal salinity can now be applied to further optimizing growth conditions in order to obtain high biomass before subjecting the algae to appropriate stress conditions for other secondary metabolite production such as glycerol, carotene, proteins or carbohydrate in the common two-phase algal cultivation system. Finally, physiological and biochemical characteristics of this strain will need to be investigated in order to assess its potential for algal biomass production and other applications such as beta-carotene, carbohydrate, lipid and protein for feed, food, aquaculture and biofuels, including providing a basis for characterizing new *Dunaliella* strains.

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