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Phylogenetic Study of Some Strains of *Dunaliella*

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ABSTRACT

Dunaliella strains were isolated from a key site for salt production in Vietnam (Vinh Hao, Binh Thuan province). The strains were identified based on Internal Transcribed Spacer (ITS) markers. The phylogenetic tree revealed these strains belong to the clades of *Dunaliella salina* and *Dunaliella viridis*. Results of this study confirm the ubiquitous nature of *Dunaliella* and suggest that strains of *Dunaliella salina* might be acquired locally worldwide for the production of beta-carotene. The identification of these species infers the presence of other *Dunaliella* species (*Dunaliella tertiolecta*, *Dunaliella primolecta*, *Dunaliella parva*), but further investigation would be required to confirm their presence in Vietnam. We anticipate the physiological and biochemical characteristics of these local species will be compared with imported strains in a future effort. This will facilitate selection of strains with the best potential for exploitation in the food, aquaculture and biofuel industries. The *Dunaliella* strains isolated and identified in this study are maintained at the Laboratory of Algal Biotechnology, International University and will be made available for research and educational institutions.

Keywords: Algae, Biotechnology, Carotene, *Dunaliella*, ITS, Phylogenetic Tree

1. INTRODUCTION

Unicellular green algae *Dunaliella* belong to the Chlorophytes (Oren, 2005). The algae was first described (Dunal, 1838), but it was not until 1905 that the name *Dunaliella* was given by Teodoresco (1905). There are currently 23 recognized species of *Dunaliella* (Pick, 1992; Oren, 2005). *Dunaliella salina* Teodoresco is the species whose vegetative cells turn yellow or orange with carotenoids in response to environmental stress such as high irradiance, high salinity, or low nutrient concentrations (Teodoresco, 1905; 1906; Lamers *et al*., 2010; Fu *et al*., 2013; Tran *et al*., 2013). However, temperature, salinity and nutrients are limiting factors on the growth and development of *Dunaliella* (Polle *et al*., 2009).

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 (Butcher, 1959; Massjuk, 1973a; 1973b). *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).

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Currently *Dunaliella* is placed in the order of *Chlamydomonadales* and the family of *Dunaliellaceae* according to the NCBI database (Polle *et al*., 2009). Because the morphology of individual species of *Dunaliella* varies considerably under different stages of growth, development and environmental conditions, it is possible that some species have been previously misidentified (Borowitzka and Borowitzka, 1988; Gonzalez *et al*., 2005; Borowitzka and Siva, 2007; Tran *et al*., 2013). According to some more recent phylogenetic studies (Gonzalez *et al*., 2001; 2005; Gomez and Gonzalez, 2004), it is believed that the total number of *Dunaliella* species may be less than 23. During the last decade, nuclear rDNA internal transcribed spacers ITS1 and ITS2 have been most commonly used to delineate *Dunaliella* species (Gonzalez *et al*., 2001; 2005; Assuncao *et al*., 2013; Tran *et al*., 2013).

Dunaliella can be found on all continents in salterns and most hypersaline lakes and in oceans all over the world. *Dunaliella* has been found in the Great Salt Lake, the Dead Sea (Oren, 2005) and in Antarctic salt lakes, other salt lakes in America, Africa, Asia, Australia and Europe (Ginzburg, 1987; Borowitzka and Borowitzka, 1988; Tran *et al*., 2013). The Vinh Hao saltern (Binh Thuan province) is a key salt producing area in Vietnam. We were attracted there to investigate *Dunaliella* biodiversity, but more specifically to search for a strain of *D. salina*. Since the regional climate of this abundant seawater resource is also conducive to high marine algal productivity we were motivated to carry out this project. Our goal was to isolate a local strain of *Dunaliella salina* to compare with strains acquired elsewhere to identify the best candidates for the exploitation of beta-carotene, carbohydrate, lipid and protein production for the food, aquaculture and biofuels industries.

2. MATERIALS AND METHODS

2.1. Sample Collection and Isolation

Algal samples were collected from salterns in Vinh Hao, Binh Thuan province. They were plated on agar medium according to Pick (1998) 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 plates after about two weeks were picked with sterile toothpicks and continuously streaked on agar petri plates until axenic alga were obtained. All cultures were maintained at room temperature (25°C), with a photon flux density of 50 µmoles photons/m^2/s.

2.2. *Dunaliella* Identification

Dunaliella were first identified based on morphological characteristics (Teodoresco, 1906; Butcher, 1959; Massjuk 1973a; 1973b; Polle *et al*., 2009). The isolates were grown in batch culture until nutrient limitation, at which *Dunaliella salina* cells would change color to yellow or orange (Polle *et al*., 2009; Lamers *et al*., 2010; Fu *et al*., 2013). The genomic DNA of these cells 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: 242 g Tris-base, 57.1 mL acetic acid, 100 mL 0.5M EDTA) and was quantified by spectrophotometer at an OD of 260 nm and stored at -20°C. Internal Transcribed Spacer (ITS) covering ITS1, 5.8S, ITS2 were PCR amplified using GoTag PCR core system II (Cat. No M7665) from Promega with pairs of primers (Forward: 5'TCCGTAGGTGAACCTGCGG3', Reverse: 5'GCATCGATGAAGAACGCAGC3'). The products were checked by electrophoresis on 1% agarose gel in 1X TAE buffer and were purified using PCR clean-up kit from Promega (Cat. No A9281).

The sequenced ITSs were aligned with respective sequences of *Dunaliella* strains obtained from NCBI (http://www.ncbi.nlm.nih.gov/) using the BioEdit program version 7.1.3.0 (Hall, 1999). 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.

3. RESULTS AND DISCUSSION

3.1. Isolation and Cell Description

Using a light microscope, various isolates grown under different salinities were scanned to match the morphological descriptions of *Dunaliella* (Teodoresco, 1906; Butcher, 1959; Massjuk, 1973a; 1973b; Polle *et al*., 2009). *Dunaliella* could not be identified in salinities of 1M, 2M, 3M. However, five *Dunaliella* isolates (A, B, C, D, E) were obtained in samples of 4M, 5M NaCl (**Fig. 1**). Generally cells of the *Dunaliella* were green, ovoid, 10 µm wide and 25 µm long. However, cells in sample A subject to nutrient limitation (**Fig. 1a**) turned orange and grew larger, presumably due to beta-carotene accumulation (**Fig. 2a**). These cells were motile with two equally long flagella in each cell (**Fig. 2a and b**) and contained a pyrenoid (d) surrounded by starch.

Fig. 1. Batch culture of the isolated *Dunaliella salina* (A) và *Dunaliella viridis* (B, C, D, E)

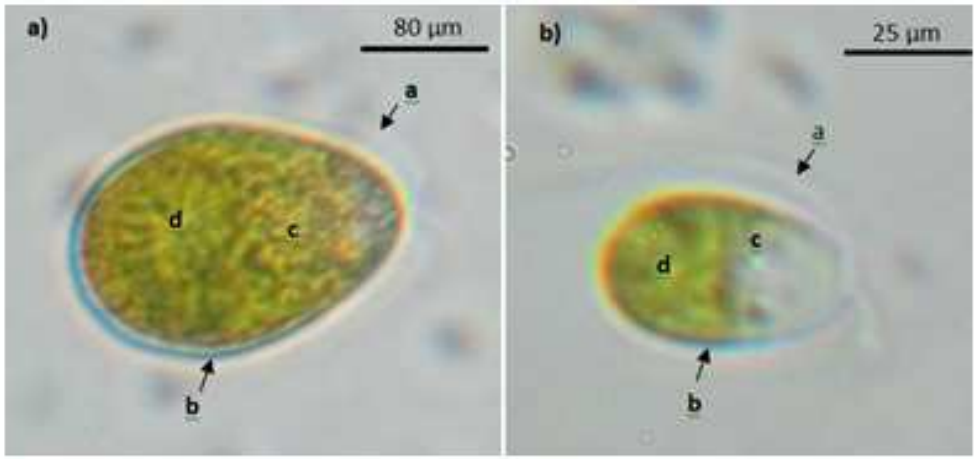

Fig. 2. Photos of *Dunaliella salina* (a) and *Dunaliella viridis* cells (b) (a). Flagella, (b). Mucilaginous layer, (c). Chloroplast, d. Pyrenoid). Note that *Dunaliella salina* cell turned orange, with larger size due to beta-carotene accumulation under nutrient limit

There was thick mucus (b) outside the cell membrane. To further confirm these isolates as *Dunaliella* and to distinguish them among the different *Dunaliella* species, it was necessary to use molecular marker ITS to delineate (Gonzalez *et al*., 2001; 2005).

3.2. Molecular Identification

A phylogenetic tree was constructed using ITS sequences from five isolates (A, B, C, D and E) and other ITS sequences data obtained from NCBI (**Table 1**). Three main clades were clearly separated (**Fig. 3**). Clade A showed isolates B, C, D and E belonging to *Dunaliella viridis* with high boottrap value (100%). Clade B contained isolate A with other strains of *Dunaliella salina and Dunaliella bardawil* from NCBI data which is believed to be a strain of *Dunaliella salina* (Polle *et al*., 2009) (Boottrap value 98%). Other *Dunaliella* species (*D. tertiolecta*, *D. parva*, *D. primolecta*) all together in clade C (boottrap value 100%), which were well separated from group of *D. salina*, *D. bardawil* and *D. viridis,* could possibly be the same species but have been wrongly named due to erroneous morphological identification (Borowitzka and Borowitzka, 1988; Gonzalez *et al*., 2005; Borowitzka and Siva, 2007). Sequences of *Chlamydomonas* obtained from NCBI were used as the out group which formed a separate clade D.

This is our first attempt to identify *Dunaliella salina* and *Dunaliella viridis* from this productive Vietnamese saltern based on morphology and molecular markers. Together, morphology and ITS sequences indicated that all isolates were *Dunaliella*, of which the isolate A was *Dunaliella salina* and the other isolates B, C, D, E were *Dunaliella viridis* (**Fig. 3**). Locally isolated *D. salina* will be compared to *D. salina* strains acquired elsewhere in order to select the best candidate for beta-carotene, carbohydrate, lipid and protein production. This future work falls outside the funding constraints of this manuscript.

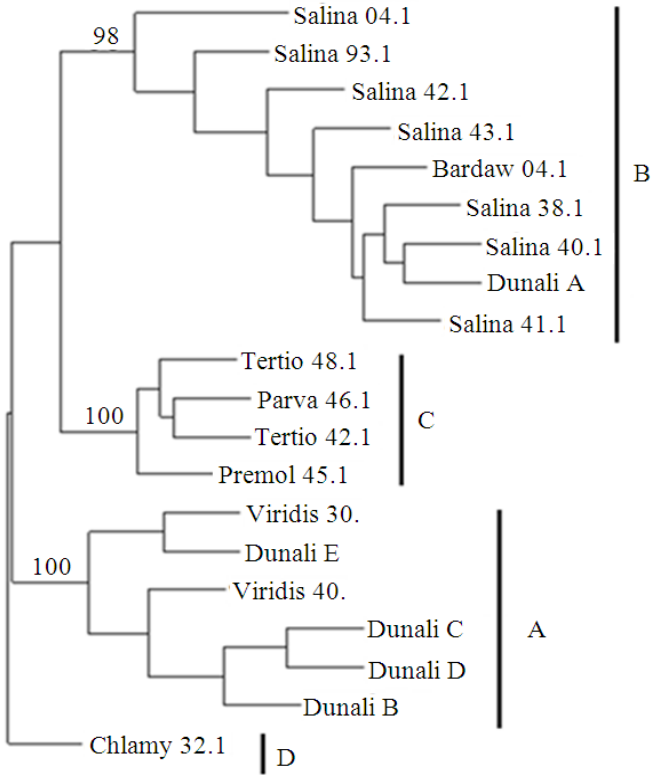

Fig. 3. Phylogenetic tree of ITS sequences of the isolated *Dunaliella salina* (clade B) and *Dunaliella viridis* (clade A). Chlamydomonas ITS sequence was used as the outgroup (clade D). All clades were separated with high bootrap value (>95%)

Table 1. ITS sequences of *Dunaliella* strains obtained from NCBI were used for building phylogenetic trees

Names	NCBI (Acc. number)	Notes
Dunaliella salina	JN797804.1	Salina 04.1
Dunaliella Salina	JX220893.1	Salina 93.1
Dunaliella Salina	DQ116742.1	Salina 42.1
Dunaliella Salina	DQ116743.1	Salina 43.1
Dunaliella Salina	DQ116738.1	Salina 38.1
Dunaliella Salina	DQ116740.1	Salina 40.1
Dunaliella Salina	DQ116741.1	Salina 41.1
Dunaliella Bardawill	JN052204.1	Bardaw 04.1
Dunaliella Tertiolecta	EF473748.1	Tertio 48.1
Dunaliella Tertiolecta	EF473742.1	Tertio 42.1
Dunaliella parva	DQ116746.1	Parva 46.1
Dunaliella primolecta	DQ116745.1	Premo l45.1
Dunaliella Viridis	HQ882840.1	Viridis 40.1
Dunaliella Viridis	HQ864830.1	Viridis 30.1
Chlamydomonas	JX839532.1	Chlamy 32.1
Dunaliella salina	Isolated	DunaliA
Dunaliella viridis	Isolated	DunaliB
Dunaliella viridis	Isolated	DunaliC
Dunaliella viridis	Isolated	DunaliD
Dunaliella viridis	Isolated	DunaliE

4. CONCLUSION

We identified *Dunaliella salina* and *Dunaliella viridis* for in Vietnam based on morphology, physiology and molecular markers. Results of this study confirm the ubiquitous nature of *Dunaliella* and suggest that strains of *Dunaliella salina* might be acquired locally worldwide for the production of beta-carotene. The identification of these species infers the presence of other *Dunaliella* species (*Dunaliella tertiolecta, Dunaliella primolecta, Dunaliella parva)*, but further investigation would be required to confirm their presence in Vietnam. We anticipate the physiological and biochemical characteristics of these local species will be compared with imported strains in a future effort. This will facilitate selection of strains with the best potential for exploitation in the food, aquaculture and biofuel industries.

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