

Full Length Research Paper

# Monitoring of uncultured *Dunaliella* sp. in an Egyptian solar saltern field based on RuBisCO-encoding gene *cbbL*

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Culture-independent molecular approach was used to explore and evaluate the diversity of *Dunaliella* species living at the salt field Malahat El-Max Alexandria, Egypt. Bulk genomic DNA was extracted directly from the collected salt water samples. Specific PCR primers and methodology were designed to amplify the gene *cbbL*, which encodes the large subunit of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) of only *Dunaliella* species, from the extracted microbial metagenome. The 700 bp-PCR amplicons were cloned and *cbbL* clone library was constructed and analyzed by sequencing. Rarefaction curve was saturated at sequence analyses of 23 clones, obtaining 19 phylotypes of *Dunaliella cbbL*, representing the total composition of *Dunaliella* in the collected sample. All recorded phylotypes had the known deduced amino acid *cbbL* motive sequence and catalytic sites. Fingerprint sequence, characterizing *Dunaliella cbbL*, was recorded. The *cbbL* phylotypes were grouped into two distinct phylogenetic clusters. The cluster 1, consisting of 18 current *cbbL* phylotypes was rooted with a cluster containing *cbbL*s of *Dunaliella salina*, *Dunaliella bioculata*, *Dunaliella primolecta* and *Dunaliella tertiolecta*. The single phylotype, uncultured *Dunaliella* EIMax.3, forming cluster 2, showed a unique phylogenetic lineage in the evolution of *Dunaliella cbbL*. This study introduced the first functional gene markers for exploring *Dunaliella* species in salt waters without culture.

**Key words:** Uncultured *Dunaliella*, RuBisCO *cbbL*, solar saltern water, diversity.

## INTRODUCTION

The genus *Dunaliella*, belonging to Chlorophyceae, has been represented by 27 known species, of which 23 are from salt water. Species of *Dunaliella* occur in freshwater, euryhaline habitats of all continents, oceans including the Dead Sea and even the salt lakes of the Antarctic (Tafreshi and Shariati, 2009). Thus, *Dunaliella* is a global genus of algae, thriving in habitats with wide ranges of salinity, pH, light intensity and temperature. The characterization of economically important algae in solar salterns has become a key focus for many research

groups all over the world (Chen and Jiang, 2009). *Dunaliella* species that are living in these fields, have an economic importance due to its highly production of anti-oxidants (Oren, 2010). In Egypt, there are several coastal solar salterns, where *Dunaliella* is living, mainly on and near to the Mediterranean coast. Studies on *Dunaliella* in Egyptian aquatic habitats have been focused on characterization, environmental stresses and biotechnological applications of isolated species, including *Dunaliella bardawil* (Adam, 1997), *Dunaliella salina* (Abd El-Baky



**Figure 1.** A map showing the sites of sampling marked by black circles.

et al., 2004; Dardir and Abd El-Tawab, 2005; Shafik, 2008), *Dunaliella parva* (Said, 2009) and *Dunaliella tertiolecta* (Tammam et al., 2011). Molecular phylogeny techniques have been applied to the taxonomic study of *Dunaliella* from 1999 onwards. These studies have encompassed the 18S rRNA genes and the internal transcribed spacer regions, ITS and have been based on gene sequence comparisons as well as on restriction fragment length polymorphism studies (Gomez and Gonzalez, 2001; Assunção et al., 2012). Little correlation was found between the molecular data and the traditional morphological-physiological attributes to delineate species within the genus *Dunaliella* (González et al., 1999, 2001).

On the basis of 18S rRNA gene sequencing, Olmos et al. (2000) could differentiate between *D. salina*, *D. parva* and *D. bardawil* as species containing one, two and three introns, respectively, within the 18S rRNA gene. Also, Hejazi et al. (2010) reported a new 18S rRNA gene arrangement in term of intron localization and nucleotide sequence in a *Dunaliella* isolated from Iranian salt lakes. However, molecular phylogenetic studies of *Dunaliella* in Egyptian solar salterns have not yet been done; remaining the diversity of *Dunaliella* species in these fields are poorly described. The gene, *cbbL* that encodes the large subunit of the enzyme RuBisCO, the key of Calvin cycle has been used as an efficient molecular tool for studying the functional diversity of several phytoplankton (Tabita, 1999; Yoon et al., 2001; Ghosh and Love, 2011). The RuBisCO large subunit is distributed in almost all phytoplankton, through five different forms: form IA, IB, IC, ID and II (Watson and Tabita, 1997; John et al., 2007). The halophylic *Dunaliella* species, the pink micro-algae, are differentiated from

other species in the class Chlorophyceae by harboring the RuBisCO form ID (Lin and Carpenter, 1997). This study developed a molecular approach for first monitoring of the diversity of *Dunaliella* based on PCR amplification, cloning and sequencing of the RuBisCO gene *cbbL* in metagenomic DNA extracted from the Saltern field, Malahat El-Max, Alexandria, Egypt. Statistical analysis was applied to determine the total composition of *Dunaliella cbbL* phylotypes and the phylogenetic tree was constructed showing the phylogenetic distribution of the recovered *Dunaliella cbbL* phylotypes.

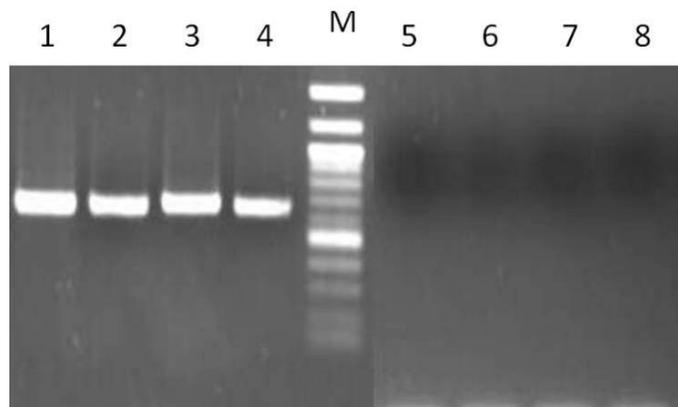
## MATERIALS AND METHODS

### Sampling

Pink-color *Dunaliella*-containing surface waters, 5 L was collected from five stations, 1 L from each station, covering mostly the total area of the saltern field, Malahat El-Max, Alexandria, Egypt (Figure 1). Each liter of the collected water was filtered on a single cylindrical 0.2 µm filter membrane unit (type Sterivex-GS, Millipore Corp., USA). In order to remove the trapped pink carotenoid pigments, which may link with the extracted DNA and hinder PCR amplification, the filters were washed with 10 ml of 80% ethanol in sterile SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-HCl, pH 7.6) several times. The inlet and outlet of the filters were capped, and the filters were stored at -30°C until processed.

### Genomic DNA extraction and amplification of the *Dunaliella* RuBisCO gene *cbbL*

Bulk microbial DNA, metagenome, was extracted essentially within the Sterivex-GS filters housing according to the method of Somerville et al. (1989), with modifications. Cell lysis and proteolysis were carried out within the filter housing using 5M guanidine



**Figure 2.** Efficiency of the PCR primers ID 537F and ID 1212R for amplification of RuBisCO form ID from genomes of known *Dunaliella* species and other phytoplankton. 1: amplification from *Dunaliella bardawil* strain TS-1; 2: *Dunaliella bioculata* CCAP 19/4; 3: *Dunaliella parva* IAM C-527; 4: *Dunaliella salina* CCAP 19/3; 5: *Chlamydomonas reinhardtii*; 6: *Synechococcus* sp. strain WH7803; 7: *Rhodospirillum rubrum*; 8: *Chlorobium tepidum*. M: 100 bp-DNA ladder marker.

thiocyanate in SET buffer at 70°C for 20 min with shake. Crude, which contained high-molecular-weight nucleic acids, was then drawn off the filter and purified using high pure PCR template preparation kit, Roche, Catalog no. 11796828001, Mannheim, Germany. The purified metagenomic DNAs, extracted from all samples, were combined and run on 0.9% agarose gel electrophoresis followed by staining with ethidium bromide and UV visualization. A new PCR primer set, Duna-537f (5'-RTW AAA CCT AAA TTA GGT YTA TCW GGT-3') and Duna-1212r (5'-AAT AGT ACC ACC ACC RAA TTG-3') were designed from multiple alignments of *cbbL* sequences of *Dunaliella* species, incorporated to date in the DNA databases, including *D. salina* strain CCAP 19/18 (acc. no. AY531529, GQ250046), *D. acidophila* (HQ142901), *D. bioculata* (AB127991), *D. primolecta* strain TS-3 (AB127992), *D. tertiolecta* (AY882012), *D. bardawil* strain TS-1 (AB127990) and *D. parva* strain UTEX 1983 (AJ001877). The primers corresponded to positions 511 to 537 (forward primer) and 1191 to 1212 (reverse primer) of the *cbbL* gene of *Synechococcus* sp. (Shinozaki et al., 1983). PCR reaction mixture (50 ml), contained 10x EX buffer II (Mg<sup>+2</sup> plus), 0.2 μM primer, 400 μM dNTP each, 2.25U Takara EX-Taq Polymerase (Takara, Japan) and 5 to 30 ng DNA template. The PCR mixture composition was performed with an initial denaturation step of 3 min at 95°C. The reaction was continued with 30 cycles of 1 min at 95°C, 2 min at 46°C, and 3 min at 72°C, with a final extension of 10 min at 72°C.

PCR products had 3'-A overhangs to facilitate TA-cloning into TOP10 *Escherichia coli* using a TOPO XL PCR-cloning kit according to the manufacturer's instructions (Catalog no. K4750-20, Invitrogen Life Technologies, Carlsbad, CA, USA). Only cells containing XL-TOPO vector with the insert were competent to grow with kanamycin. Colonies of these cells were screened directly by sequencing using vector primers T7 and an ABI 3730xl 96-capillary DNA analyzer (Applied Biosystems, Foster City, CA, USA).

#### Sequence and bioinformatics analyses of *cbbL* genes

Sequencing results were introduced to FASTA (<http://fasta.ddbj.nig.ac.jp/top-e.html>), to determine their similarity to

known *Dunaliella cbbL* sequences, deposited in DNA database. Nucleotide sequences for *cbbL* open reading frames (ORFs) were submitted to Transeq, (<http://www.ebi.ac.uk/emboss/transeq/>), to obtain the inferred amino-acid sequences. The correct *cbbL* ORFs were identified from the presence of diagnostic *cbbL* CO<sub>2</sub> fixation motifs. Grouping of sequences into phylotypes was done based on genetic distances using the Mothur software package V.1.7.2 (Schloss et al., 2009), where *cbbL* sequences, having 100% deduced amino acid identities, were grouped into a single phylotype. The expected total composition of *Dunaliella cbbL* was determined by rarefaction analyses in the Mothur software package. The phylogenetic analysis was done based on deduced amino acid and nucleotide sequences, and included the current *cbbL* sequences and their *Dunaliella cbbL* homologues, with significant homologies, *e*-value < 0.001, from the DNA database bank. The process was performed by applying the neighbor-joining algorithm and drawing the trees using the MEGA 3.1 software (<http://www.megasoftware.net/>). The branching patterns of the constructed phylogenetic tree were confirmed by reconstruction of the phylogenies using two other methods of analysis, namely maximum parsimony and maximum-likelihood, contained within the Phylip package.

The sequences of recovered *Dunaliella cbbL* were deposited in DNA data base bank under accession numbers from AB781569 to AB781587.

## RESULTS AND DISCUSSION

### Efficiency of primers for PCR amplification of *Dunaliella cbbL*

This work aimed to cover the molecular diversity of almost all *Dunaliella* species in the collected saline water sample. In order to achieve this target, several considerations were involved in the design of an efficient primer set for amplification of *Dunaliella cbbL*. The primers must be designed from *cbbL* region, which encodes CO<sub>2</sub> catalytic site of *Dunaliella* RuBisCO form ID. Hence, the codon region of the *cbbL* catalytic site amino acid group T(V) KPPLG (Newman and Gutteridge, 1993) represented specific sequence for the design of the primer Duna-537f. The primer Duna-1212r was designed from the codons that encode the catalytic amino acids QFGGGTI, characterizing the *Dunaliella* RuBisCO ID *cbbL*. These primer sequences were differed from other *cbbL* sequences, located in other autotrophic algae, in several nucleotides, sufficient to make each of the primers binds only with *Dunaliella cbbL*. This was clear in the positive PCR amplifications of *Dunaliella cbbL*, while negative amplifications were noticed for *cbbL* from other phytoplankton species (Figure 2). These primer sets provided *cbbL* product size, 700 bp, longer than that produced by Paul et al. (2000). This gave an extended view to screen the diversity of *Dunaliella cbbL*.

In order to reduce the possibility of bias in amplification of *Dunaliella cbbL*, the PCR was tested using a range from 26 to 30 cycles, and then the amplicons were combined for cloning (Suzuki and Giovannoni, 1996). This was clear in the rarefaction curves, for the recovered *Dunaliella cbbL*, which showed saturation after analysis of 23 clones, yielding 19 phylotypes (Supplementary

		K	K		G	X	D	F	X	K	
Uncultured <i>Dunaliella</i>	ElMax-1	GTAA <b>AAA</b> CCT <b>AAA</b> TTAGGTTTATCTGGT	27	TGGATTAGACTTTACCAAA	84						
Uncultured <i>Dunaliella</i>	ElMax-2	.....C.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-3	.....T.....	27	G..T.....T..C..T..G	84						
Uncultured <i>Dunaliella</i>	ElMax-4	.....	27	T..A.....C..T..C..A	84						
Uncultured <i>Dunaliella</i>	ElMax-5	.....	27	.....G..G	84						
Uncultured <i>Dunaliella</i>	ElMax-6	.....	27	.....C..A	84						
Uncultured <i>Dunaliella</i>	ElMax-7	.....C.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-8	.....T.....	27	.....G..G	84						
Uncultured <i>Dunaliella</i>	ElMax-9	.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-10	.....C.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-11	.....T.....	27	.....C..	84						
Uncultured <i>Dunaliella</i>	ElMax-12	.....C.....	27	.....G..	84						
Uncultured <i>Dunaliella</i>	ElMax-13	.....	27	.....G.G	84						
Uncultured <i>Dunaliella</i>	ElMax-14	.....	27	.....CA.A	84						
Uncultured <i>Dunaliella</i>	ElMax-15	.....T.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-16	.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-17	.....	27	.....	84						
Uncultured <i>Dunaliella</i>	ElMax-18	.....	27	.....T..	84						
Uncultured <i>Dunaliella</i>	ElMax-19	.....	27	.....A..	84						
<i>Dunaliella bioculata</i>		A.T.....A.....A..	534	.....G..G	591						
<i>Dunaliella primolecta</i>		.....	534	.....	591						
<i>Dunaliella tertiolecta</i>		.....	547	.....	603						
<i>Dunaliella bardawil</i>		.....	531	.....C..A	588						
<i>Dunaliella salina</i>		.....	504	...T.....G..G	561						
<i>Dunaliella parva</i>		.....	516	.....C..T..	573						
<i>Chlamydomonas reinhardtii</i>		..C...A.T...C.C...C.T.....	546	...C.T.....A	603						
<i>Synechococcus</i> sp.		.....G..G..G.....C..GAGC..C	522	C.....G.....C..G	579						
<i>Rhodospirillum rubrum</i>		.....C.TCCC	519	...--C.....T....	573						

**Figure 3.** Multiple alignments, within the amplification size, 700 bp, of recorded uncultured *Dunaliella* ElMax.1 to 19, and *cbbL*s from other known phytoplankton recorded in database. The nucleotide alignments are only in the sites of RuBisCO *cbbL* motive sequence, marked by shading, and catalytic amino acids, bold face. Alignments in the box represent the finger print sequence of *Dunaliella cbbL*. Species used in the alignments had accession numbers listed in Figure 4.

Figure 1). This result may reflect the efficiency of the designed primers and PCR methodology in covering the total composition of *Dunaliella* in the studied water sample.

#### The recovered *cbbL* phlotypes were belonged to uncultured *Dunaliella* species

All recovered *Dunaliella cbbL* phlotypes were from metagenomic DNAs and, consequently, the source *Dunaliella* species were uncultured and had no common synonyms. Hence, nomenclatures were given to these phlotypes, whereby each phlyotype was the descriptor of uncultured *Dunaliella* followed by clone name, which included sampling site name and clone number, for example, uncultured *Dunaliella* ElMax.1 and so on. *cbbL* phlotypes have been recovered from uncultured autotrophic microbial communities, which have no obvious species identifications (Guo et al., 2013).

#### The recovered phlotypes had the known *cbbL* deduced amino acid catalytic sites, beside specific sequence characterizing *Dunaliella cbbL*

Alignments of 702 bp-*cbbL* phlotypes, which yielded 234 deduced amino acids, were done with those recovered from known *Dunaliella* species (Figure 3). The positions of catalytic sites were corresponded to the *cbbL* of *Synechococcus* sp. (Newman and Gutteridge, 1993). All the current *Dunaliella cbbL* phlotypes possessed the characteristic RuBisCO motif sequence, GXDFXKXDE. Each of the amino acids GDFKE in the motif sequence was represented by two codons, for example, the amino acid G was represented by the codon "GGT" in the phlyotype uncultured *Dunaliella* ElMax.3 and the codon "GGA" in other recovered phlotypes (Figure 3). The recovered *cbbL* phlotypes contained the known *cbbL* catalytic deduced amino acid sequences. The nucleotide positions from 714 to 741, of *cbbL* of *Synechococcus* sp. were found to contain the finger print sequence, 5'-

	K	S	G	G	G
Uncultured <i>Dunaliella</i> ElMax-1	AAA 483	TCTGGTGGT	624	GGTGGT	693
Uncultured <i>Dunaliella</i> ElMax-2	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-3	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-4	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-5	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-6	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-7	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-8	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-9	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-10	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-11	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-12	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-13	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-14	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-15	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-16	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-17	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-18	... 483	.....	624	.....	693
Uncultured <i>Dunaliella</i> ElMax-19	... 483	.....	624	.....	693
<i>D. bioculata</i>	... 990	.....	1131	.....	1200
<i>D. primolecta</i>	... 990	.....	1131	.....	1200
<i>D. tertiolecta</i>	... 1002	.....	1143	.....	1212
<i>D. bardawil</i>	... 987	.....	1128	.....	1197
<i>D. salina</i>	... 960	.....	1101	.....	1170
<i>D. parva</i>	... 972	.....	1111	-----	
<i>C. reinhardtii</i>	... 1002	..A..C..	1143	.....	1212
<i>Synechococcus</i> sp.	..G 978	..C.....	1119	.....	1189
<i>R. rubrum</i>	... 980	AG.....C	1110	..C..C	1182

Figure 3. Contd.

GCTGAAGGAATGCTTCAACGTGCACAA-3', of the *Dunaliella cbbL* (Figure 3). Consequently, the amino acids AGLQQ (Figure 3), encoded by this finger print nucleotide sequence, were considered as *Dunaliella* RuBisCO key amino acids, which differentiate it from those of in other phytoplankton. *cbbL* finger print sequences have been recorded for several autotrophic bacterial endosymbionts and used as nucleotide probes for *in situ* localization of the corresponding *cbbL* gene in the host tissues (Elsaied et al., 2002, 2006; Kimura et al., 2003).

On the other hand, red-like RuBisCO *cbbL* marker sequence has been used as a TaqMan probe for detection and quantification of soil autotrophic bacteria (Selesi et al., 2007). Hence, this current *Dunaliella cbbL* finger print sequence may be used as a biomarker for detection of uncultured *Dunaliella* by probing and hybridization techniques.

### The recovered *Dunaliella cbbL* phylotypes were phylogenetically unique

Phylogenetic tree of *cbbL* was constructed in order to demonstrate the classification of the recovered *Dunaliella cbbL* (Figure 4). The nineteen *cbbL* phylotypes were distributed in two unique phylogenetic clusters. The cluster 1 harbored 18 *cbbL* phylotypes, which showed an average value of 95%<sub>ND</sub> (nucleotide identity), that is, 97%<sub>aa</sub> (amino acid identity) with each others. On the other hand, the *cbbL* phylotypes, constituting cluster 2, were distinct from other known *Dunaliella* species located in the same *Dunaliella* phylogenetic branch by showing homologies of 89 to 90%<sub>ND</sub> (92 to 93%<sub>aa</sub>) (Figure 4). The uncultured *Dunaliella* ElMax.3, forming the cluster 2, had a unique *cbbL* phylogenetic lineage. It showed average identity of 87%<sub>ND</sub> (89%<sub>aa</sub>) with other current *Dunaliella*

	K	K		G	X	D	F	X	K	
D-1	<b>GTAAAA</b>	<b>CCCTAA</b>	<b>ATTAGG</b>	<b>TTTATCT</b>	<b>GGT</b>	27	<b>TGGATTAGACTTTACCAAA</b>	84		
D-2						27				84
D-3						27				84
D-4						27				84
D-5						27				84
D-6						27				84
D-7						27				84
D-8						27				84
D-9						27				84
D-10						27				84
D-11						27				84
D-12						27				84
D-13						27				84
D-14						27				84
D-15						27				84
D-16						27				84
D-17						27				84
D-18						27				84
D-19						27				84
<i>Dunaliella bioculata</i>	A.T.	A.			A.	534				G.G 591
<i>Dunaliella primolecta</i>						534				591
<i>Dunaliella tertiolecta</i>						547				603
<i>Dunaliella bardawil</i>						531				C.A 588
<i>Dunaliella salina</i>						504				T.G.G 561
<i>Dunaliella parva</i>						516				C.T. 573
<i>Chlamydomonas reinhardtii</i>	C.	A.T.	C.C.	C.T.		546				C.T. A 603
<i>Synechococcus sp.</i>		G.G.G.		C.GAGC.	C	522				C.G 579
<i>Rhodospirillum rubrum</i>						519				C.TCCC T. 573

	X	D	E		A	E	G	M	L	Q	R	A	Q		H	R	H	
D-1	<b>GATGACGAA</b>			93	<b>GCTGAAGGAATGCTTCAACGTGCACA</b>									246	<b>CACCGT</b>	366	<b>CAC</b>	462
D-2				93										246		366		462
D-3			G	93			CG		T	AGT				246		366		462
D-4			A	93			GA		C	TCA				246		366		462
D-5				93										246		366		462
D-6				93										246		366		462
D-7				93										246		366		462
D-8				93										246		366		462
D-9				93					C					246		366		462
D-10				93					T					246		366		462
D-11				93										246		366		462
D-12				93										246		366		462
D-13				93										246		366		462
D-14				93										246		T. 366		462
D-15				93										246		366		462
D-16				93										246		366		462
D-17				93										246		C. 366		462
D-18				93										246		366		462
D-19				93										246		366		462
<i>D. bioculata</i>				600										753		873		969
<i>D. primolecta</i>				600										753		873		969
<i>D. tertiolecta</i>				612										765		885		981
<i>D. bardawil</i>				597										750		870		966
<i>D. salina</i>				570										723		843		939
<i>D. parva</i>				582										735		855		951
<i>C. reinhardtii</i>				612										765		885		981
<i>Synechococcus sp.</i>				588										741		C 861		957
<i>R. rubrum</i>				582										737		G 864		965

**Figure 4.** A tree shows the phylogenetic relationship between the current uncultured *Dunaliella* clones, EIMax.1 to 19, marked with bold face, and other *cbbL* sequences from *Dunaliella* species available in database, beside *cbbL* sequences from other phytoplankton, as out groups. The consensus tree was constructed by neighbour-joining analysis and confirmed by maximum-parsimony and maximum-likelihood methods. Bootstrap values of >50% are indicated at the nodes. The bar represents 0.1 changes per amino acid or nucleotide.

	K	S	G	G	G
D-1	AAA 483	TCTGGTGGT	624	GGTGGT	693
D-2	... 483	.....	624	.....	693
D-3	... 483	.....	624	.....	693
D-4	... 483	.....	624	.....	693
D-5	... 483	.....	624	.....	693
D-6	... 483	.....	624	.....	693
D-7	... 483	.....	624	.....	693
D-8	... 483	.....	624	.....	693
D-9	... 483	.....	624	.....	693
D-10	... 483	.....	624	.....	693
D-11	... 483	.....	624	.....	693
D-12	... 483	.....	624	.....	693
D-13	... 483	.....	624	.....	693
D-14	... 483	.....	624	.....	693
D-15	... 483	.....	624	.....	693
D-16	... 483	.....	624	.....	693
D-17	... 483	.....	624	.....	693
D-18	... 483	.....	624	.....	693
D-19	... 483	.....	624	.....	693
<i>D. bioculata</i>	... 990	.....	1131	.....	1200
<i>D. primolecta</i>	... 990	.....	1131	.....	1200
<i>D. tertiolecta</i>	... 1002	.....	1143	.....	1212
<i>D. bardawil</i>	... 987	.....	1128	.....	1197
<i>D. salina</i>	... 960	.....	1101	.....	1170
<i>D. parva</i>	... 972	.....	1111	-----	
<i>C. reinhardtii</i>	... 1002	..A..C...	1143	.....	1212
<i>Synechococcus</i> sp.	..G 978	..C.....	1119	.....	1189
<i>R. rubrum</i>	... 980	AG.....C	1110	..C..C	1182

Figure 4. Contd.

*cbbL* phlotypes and those of known *Dunaliella* species.

These results may indicate that dynamically studied solar saltern field may select for different and diverse uncultured *Dunaliella* lineages, which added to *Dunaliella* phylogeny. In conclusion, this study could monitor uncultured *Dunaliella* based on specific *cbbL*-PCR approach. This strategy will help in finding diverse *Dunaliella* species yet to be discovered and open a new window to study the phylogeny of uncultured *Dunaliella* in aquatic environments.

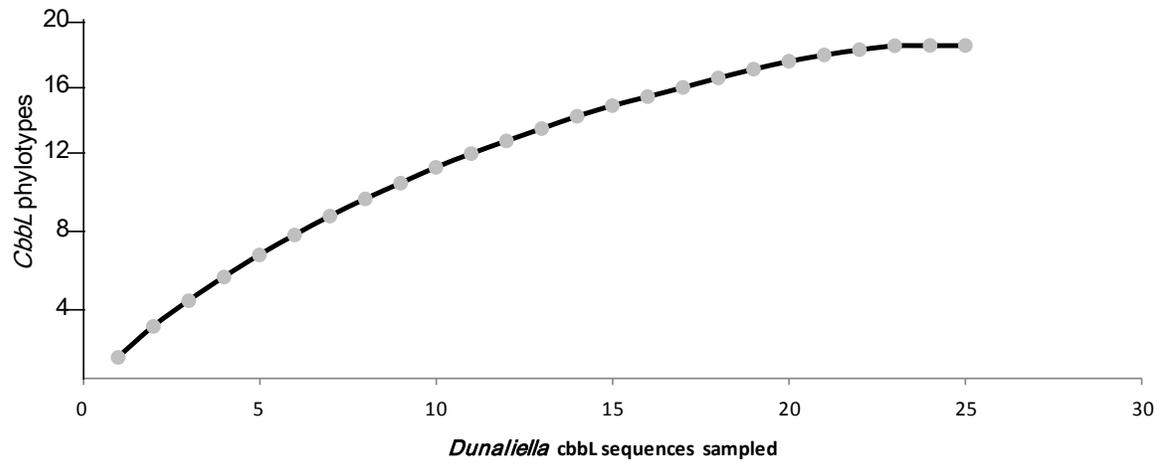
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**Supplementary Figure 1.** Rarefaction curves for the expected number of *Dunaliella cbbL* gene phylotypes.