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ORIGINAL PAPER

A novel subaerial *Dunaliella* species growing on cave spiderwebs in the Atacama Desert

A. Azúa-Bustos · C. González-Silva · L. Salas · R. E. Palma · R. Vicuña

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Abstract Strategies for life adaptation to extreme environments often lead to novel solutions. As an example of this assertion, here we describe the first species of the well-known genus of green unicellular alga *Dunaliella* able to thrive in a subaerial habitat. All previously reported members of this microalga are found in extremely saline aquatic environments. Strikingly, the new species was found on the walls of a cave located in the Atacama Desert (Chile). Moreover, on further inspection we noticed that it grows upon spiderwebs attached to the walls of the entrance-twilight transition zone of the cave. This peculiar growth habitat suggests that this *Dunaliella* species uses air moisture condensing on the spiderweb silk threads as a source of water for doing photosynthesis in the driest desert of the world. This process of adaptation recapitulates the transition that allowed land colonization by primitive plants and shows an unexpected way of expansion of the life habitability range by a microbial species.

Keywords *Dunaliella* · Atacama Desert · Evolution · Cave · Adaptations · Water

Abbreviations

TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
CLSM	Confocal laser scanning microscopy
a.s.l.	Above sea level

Introduction

The genus *Dunaliella* (Chlorophyta, Chlorophyceae, Chlamydomonadales, Dunaliellaceae) comprises a group of aquatic unicellular green algae proposed to account for most of the primary production in hyper saline watery environments around the world (Oren 2005). Besides hyper saline lagoons and lakes, some species are also found in marine environments (Berden-Zrimec et al. 2008; Colomb et al. 2008), whereas others may also be found in the water-saturated shores of saline ponds (Borowitzka and Silva 2007). Since 1905, about 28 species of this genus have been described (Borowitzka and Silva 2007; González et al. 2009), being the hyper salinity tolerant *Dunaliella salina* Teodor, the first and best known described species (Teodoresco 1905). *Dunaliella* cells are ovoid to pyriform in shape, lack a rigid cell wall and are enclosed by a thin

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A. Azúa-Bustos (✉) · L. Salas · R. Vicuña
Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
e-mail: ajazua@uc.cl

C. González-Silva
Centro de investigación del Medio Ambiente (CENIMA), Universidad Arturo Prat, Iquique, Chile

A. Azúa-Bustos · R. Vicuña
Millennium Institute of Fundamental and Applied Biology (MIFAB), Santiago, Chile

R. E. Palma
Departamento de Ecología y Centro de Estudios Avanzados en Ecología y Biodiversidad, CASEB, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

plasma membrane that causes the cells to round up as the external salinity drops down (Oren 2005). Their size varies from 5 to 25 μm in length and from 3 to 13 μm in width. The cells contain a single cup-shaped chloroplast with a central pyrenoid usually surrounded by an amylosphere of starch grains (Ben-Amotz 1980; Borowitzka and Silva 2007). *Dunaliella* cells have the organelles typical of green algae, i.e., membrane-bound nucleus, mitochondria, Golgi apparatus, vacuoles and an eyespot (Ben-Amotz and Avron 1989). During their life cycle, some *Dunaliella* species may also develop a vegetative "palmella" or "palmelloid" stage formed by a colony-like group of round non-motile cells found in benthic algal mats coating rocks and other solid submerged substrates (Brock 1975; Borowitzka and Silva 2007). They reproduce either by longitudinal division of biflagellate motile cells or by fusion of two motile cells to form a zygote (Oren 2005). Being an obligate photoautotroph, in addition to chlorophylls *a* and *b*, *Dunaliella* cells contain several carotenoid pigments such as α and *β* carotene, neoxanthin, lutein, violaxanthin and zeaxanthin, which contribute to prevent chlorophyll photo-damage (Hosseini Tafreshi and Shariati 2009). Under specific conditions (high light intensity exposure, nutrient limitation, etc.), some *Dunaliella* species synthesize *β*-carotene, which accumulates as granules between the thylakoids in the cell's chloroplast, thus providing them with a bright red color (Aasen et al. 1969). *Dunaliella* is halotolerant, constituting a model organism for salt adaptation. It can withstand NaCl concentrations ranging from about 0.05 M up to saturation (5.5 M) (Liska et al. 2004). One of the mechanisms for salt adaptation is the accumulation of photosynthetically produced glycerol, which acts as an osmotic compatible solute (Chen and Jiang 2009). In cells grown in 4 M NaCl, glycerol accumulation can reach as high as 7.8 M, equivalent to a solution of 718 g/l glycerol in water (Borowitzka and Brown 1974; Brown 1990). Under salt stress, *Dunaliella* cells also produce high amounts of extracellular polymeric substances (EPS) that can reach up to 944 mg/l when grown in 5 M NaCl (Mishra and Jha 2009). In addition to the reported halotolerance of most *Dunaliella* species, *Dunaliella acidophila* is able to grow in highly acidic environments (pH 0–1), some strains of *Dunaliella salina* tolerate high light intensities and a *Dunaliella* species found in Antarctica can endure subzero temperatures (Hosseini Tafreshi and Shariati 2009). Furthermore, *Dunaliella* is more tolerant to fuel oil contamination compared with other 66 planktonic algae (Brown and Borowitzka 1979). Thus, *Dunaliella* species are unique in their abilities to adapt to extreme environments.

The above characteristics constitute the basis for several biotechnological applications, such as the industrial production of *β*-carotene, glycerol, lipids, vitamins, minerals and proteins. This alga also has an interesting potential for foreign protein expression, the production of bioindicators and biofuels, wastewater treatment, etc. (Borowitzka et al. 1984; Hosseini Tafreshi and Shariati 2009; Gouveia and Oliveira 2009).

In this work, we report a novel species of *Dunaliella* that evolved to thrive outside an aquatic environment. This microalga was found growing onto spiderwebs inside a cave in the Atacama Desert, the driest and oldest desert of the world (Houston and Hartley 2003; Hartley et al. 2005) and a well known Mars analog model in astrobiology. The identification as a novel species was confirmed by means of both molecular and morphological methods.

Materials and methods

Relative humidity (RH) at the cave interior was measured with miniature sized loggers (16 mm diameter × 6 mm height) (Maxim Integrated Products, Inc) placed behind a colonized spiderweb, taking continuous readings every 10 min for 30 days (November 2008). For Photosynthetic Photon Flux Density (PPFD) measurements, the sensor (Apogee Quantum Meter QMSW-SS calibrated for sunlight) was placed parallel to the colonized spiderwebs and then pointed to the entrance.

Microscopy; TEM, SEM and CLSM were as previously detailed (Azua-Bustos et al. 2009).

Red ruthenium staining

Cells were fixed with 3% glutaraldehyde in sodium cacodylate buffer 0.132 M pH 7.2 containing 1 mg/ml of red ruthenium for 4 h. After a 2-h wash with sodium cacodylate buffer 0.132 M pH 7.2, the samples were treated with 1% aqueous osmium tetroxide during 90 min. The samples were then briefly washed with distilled water and dehydrated with a graded ethanol series (50–100%), 1:1 ethanol/acetone and acetone 100% 5 min for each concentration used. Samples were preembedded overnight with epon–acetone 1:1 and then embedded in pure epon. The polymerization process was done at 60°C for 24 h. Sixty-nano-thin sections were obtained with a Sorvall MT-5000 ultramicrotome and stained with 4% uranyl acetate in methanol for 2 min and lead citrate for 5 min. Observations were made with a Philips Tecnai 12 BioTwin transmission electron microscope operated at 80 kV.

Phylogenetic characterization

Total genomic DNA was extracted from 100 mg of alga-covered spiderwebs using a Soil DNA Isolation Kit (MoBio Laboratories). The 18S rRNA nuclear gene was amplified using previously published *Dunaliella* specific primers

(Olmos et al. 2000). The 16S rRNA chloroplast gene was amplified using the primers Dun16SFw42: 5′-CGATC AGTAGCTGGTTTGAGAG-3′; Dun16SRv688: 5′-TTCT TTGCGTTGCATCAAATT-3′; Dun16SRv784: 5′-CAGC CATGCACCACCTGTGTT-3′; Dun16SFw595: 5′-ACG CGTTAAGTTTCCCGCCTG-3′; Dun16SRv1070: 5′-GCG ATTACTATAGATTCCGGCT-3′ and Dun16SRv1102: 5′-CAGGAACGTATTCACCGC-3′. The *psa*B chloroplast gene, coding for the photosystem I P700 chlorophyll *a* apoprotein A2, was amplified using the primers DunpsaBFw22: 5′-ATTTGGGATCCACATTTTGGT-3′; DunapsaBRv753: 5′-TACTGAAGCTAAAGCTAAA-3′; DunpsaBfw548: 5′-TTTATGGTTAACAGATATGGC-3′ and DunpsaBRv1269: 5′TCCAATAGTTAAGAATA AAGA3′. The *rbcL* plastid gene coding for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit was amplified using *rbcL* specific primers rbcl-Dun-Fw1 5′-GC TTTCCGTATGACACCTCAA-3′ and rbcl-Dun-Rv2 5′-A GTTTTAGCTAAAACACGGAA-3′. These primers were designed based of sequence comparisons of known *rbcL*, 16S rRNA and *psa*B *Dunaliella* genes. Amplification conditions were as previously reported (Azua-Bustos et al. 2009). Automated sequencing was done by Macrogen DNA Sequencing Inc. (Seoul, Korea).

The nucleotide sequences of the *Dunaliella atacamensis* 18S rRNA, 16S rRNA, *psa*B and *rbcL* genes were analysed using the Megablast option for highly similar sequences of the BLASTN algorithm against the NCBI non-redundant database. A multiple alignment was then performed using CLUSTALW. Accession numbers for the 18S rRNA, 16S rRNA, *psa*B and *rbcL* genes of *Dunaliella atacamensis* are FJ917192, HM126014, HM126015 and FJ985682, respectively. For the 18S rRNA gene, phylogenetic reconstructions were performed through neighbour joining and maximum parsimony using PAUP* 4.0b10 (Swofford 2002). For parsimony, all characters were analysed as unordered with four possible states (*A*, *C*, *G* and *T*), excluding phylogenetically uninformative characters. The equally parsimonious trees were found using heuristic search, with the branch swapping option and the TBR swapping algorithm available in PAUP*. The neighbour joining tree was obtained through the distance matrix considering absolute values. Nodes support was accomplished through non-parametric bootstrap with 1000 replicates for both parsimony and distance. Trees were rooted with the outgroup criterion using the published sequence of the *Asteromonas gracilis* strain CCMP 813 18S rRNA gene.

Both, for the 16S rRNA and the *rbcL* genes, phylogenetic reconstructions were performed through neighbour joining using PAUP*. The neighbour joining tree was obtained through the distance matrix considering absolute values, and node support was accomplished through non-parametric bootstrap with 5000 replicates. The tree was rooted with the outgroup criterion using the published sequence of the *Chlamydomonas reinhardtii* 16S rRNA gene. All 16S rRNA *Dunaliella* genes sequences available in Genbank which aligned with our sequence were used. For the *psaB* gene, the phylogeny was performed through maximum likelihood (ML) using PAUP*. We selected the best-fitting model of nucleotide substitution using the corrected Akaike Information Criterion (AIC; Akaike 1974). We evaluated support for the nodes with 1000 bootstrap replicates (Felsenstein 1985). The AIC criterion identified the GTR + I + G as the most likely model of base pair substitution, and the invariant (I) sites were 0.5319, the gamma shape parameter was = 1.6872 and base frequencies were $A = 0.2429$, $C = 0.1783$, $G = 0.2019$ and $T = 0.3769$. We rooted the tree with the outgroup criterion, using the published sequence of the *Chloromonas radiata* strain UTEX 966 *psaB* gene.

Pigment identification

Pigment extraction; A 2 ml aqueous suspension of cells was centrifuged at 14000 rpm for 2 min. 200 µl of 90% acetone was added to the pellet and vortexed at maximum speed for 10 min. After a 1-min centrifugation at 14000 rpm the pellet was discarded and the supernatant was measured using a Shimadzu UV-160 spectrophotometer after previous dilution of 1:5 in acetone.

Results and discussion

Habitat description

The species of *Dunaliella* herein described was found inside a cave located 107 km off the city of Iquique, Chile, in the Coastal Range hills of the Atacama Desert. This is a very arid region, averaging 0.8 mm of annual rainfall over the past 30 years at the coastline (12 m a.s.l.). The mean annual temperature in this area is 18°C, the average annual maximum is 22°C, the average annual minimum is 16°C and the average RH is 68%. Temperatures at higher elevations (515 m a.s.l.) are cooler by almost 4°C, while the humidity exceeds 75% (Cereceda et al. 2007). The cave, situated about 75 m a.s.l., is about 170 m deep and has an average height of 50 m. Its entrance directly confronts the Pacific Ocean, which is 154 m away from it. Here, the arid Coastal Range that separates the hyperarid Atacama Desert plateau from the Pacific Ocean acts as a topographic barrier to clouds and moisture-rich marine air moving north and north-eastwards from the ocean. Thus, fog-originated water allows the presence of "fog oases" in this region that sustain a number of endemic plant species and is even being proposed as a source for human consumption

(Rundel et al. 1990; Kraus et al. 2001; Espejo 2001; Cereceda et al. 2002; Larrain et al. 2002; Osses et al. 2005). These clouds show a daily cycle with a maximum expansion over the coastal hills during the night and early morning hours (Farías et al. 2005; Cereceda et al. 2008). This causes the cave interior to act like a funnel that continuously captures the incoming water rich air of oceanic origin, in particular the south-facing walls exposed to the moisture rich prevailing winds.

Not surprisingly, the alga grows only on the south-facing walls of the cave and about 20 m away from the entrance. PPFD values measured at the site of colonization were of 1.4% of the outside incident light. In closer inspection we noted that most of the alga grows as colonies of green cells covering spiderwebs attached to the cave walls and not on the underlying rocks (Fig. 1a, Movie S1). The colonized spiderwebs are constantly subjected to the flow of the humid rich air moving through them, condensing water droplets observed in the early hours of the morning (Fig. 1c, d, Movie S1). In agreement with this, by placing a RH microsensor inside the cave directly behind a spiderweb attached to the wall, we recorded daily variations in air RH in the range of 14–73%, with the higher values observed during the night and early hours of the morning (Fig. S1). These RH values agree with those measured by Cereceda et al. (2007) in nearby areas located between 12 and 515 m a.s.l.

Condensation of water onto the hygroscopic threads of spiderwebs is a well-known phenomenon (Vehoff et al. 2007). Therefore, the unusual growth habitat in the context of the driest desert of the world suggests that this species of *Dunaliella* evolved to use air moisture captured by condensation on the spiderweb silk (Movie S1). Mean annual fog water yields using standard fog collectors for a site 50 km north of the cave have been measured to be less than 0.02 l^{-1} m^{-2} (Cereceda et al. 2008). Although low, these levels imply a daily availability of a thin layer of water that can sustain photosynthetic microbial communities as shown in this work. An alternative source of water at this site could be dew. Spontaneous droplet formation is induced by substrates with temperatures below the dew point of the ambient air. As the exposed surfaces cool by radiating heat during the late afternoon and night, atmospheric moisture could condense at a rate greater than that at which it can evaporate, resulting in the formation of water droplets on the spiderweb threads at the early hours of the morning as observed. The poor thermal conductivity of the spiderweb threads (Osaki 1989) may add to this process.

Interestingly, sodium levels reported in fog water collected in nearby areas suggest that this element comes

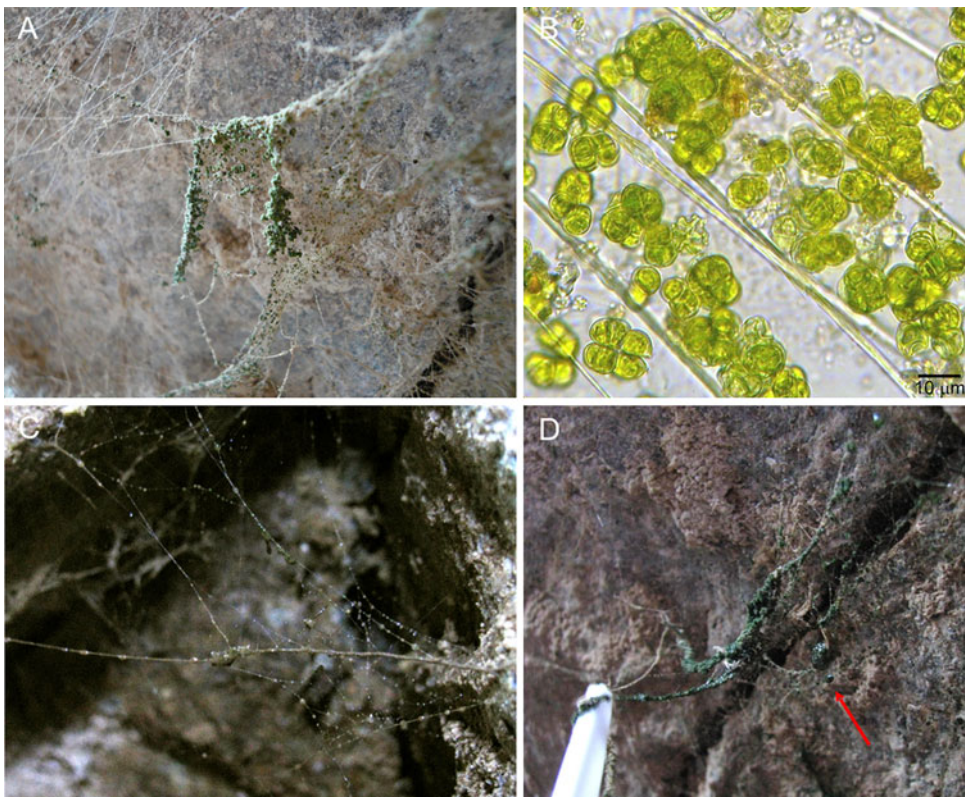

Fig. 1 Habitat description. **a** Colonies of *Dunaliella atacamensis* cells growing onto spiderwebs attached to the cave walls. Note that no growth is observed on the underlying rocks. **b** Bright field micrograph of colonies of *Dunaliella* cells. The silk threads of the spiderweb can also be seen. **c** Water condensation on the spiderweb silk threads as seen at 6:30 a.m. **d** Water condensation on the colonized spiderweb seen in **a**. The *red arrow* points to a colony of cells immersed in a water droplet

mainly from the sea (Cáceres et al. 2004), providing a potential source of origin for the reported cave inhabiting *Dunaliella* species.

Species identification

Eukaryota; Viridiplantae; Chlorophyta; Chlorophyceae; Chlamydomonadales; Dunaliellaceae; *Dunaliella atacamensis*. *D. atacamensis* has individual and uninucleate non motile cells. Algae with rigid cell walls, which may attain considerable thicknesses (0.5 μm). Cells have a light green-emerald colour and are mostly round or ovoid, with a diameter of 6 μm. *D. atacamensis* has one cup-shaped chloroplast occupying about half of the cell interior, and a single pyrenoid of about 0.7 × 1 μm surrounded by several starch bodies. No flagellum appears to be present, although small stub-like structures suggestive of flagella were observed in one of the few single cells found. The cells form tetrads which join into colony-like structures in irregular clumps reminiscent of palmelloid stages of other *Dunaliella* species. Daughter cells are of similar size after division.

The identity of this alga as a new species of *Dunaliella* was confirmed by both molecular and morphological methods. Molecular techniques have proved useful in taxonomic studies of *Dunaliella* species, particularly when using 18S rRNA and internal transcribed spacer (ITS) regions specific oligonucleotides (González et al. 1999; González et al. 2001; Olmos-Soto et al. 2002; Gómez and González 2004; Raja et al. 2007). In the present work, these 18S rRNA specific primers amplified the expected 1771-bp genomic product. This was further confirmed by using *Dunaliella* 16S rRNA and *psa*B specific primers, which amplified 152 and 646 bp plastid products, respectively.

Automated sequencing followed by BLAST search, alignment and phylogenetic analyses of the 18S rRNA, 16S rRNA and *psa*B genes revealed a species of *Dunaliella* different from those previously described. Both neighbour joining (NJ) and maximum parsimony (MP) strict consensus tree obtained from the aligned 18S rRNA nuclear gene sequences of 13 *Dunaliella* strains confirmed *Dunaliella atacamensis* as a new member of the *Dunaliella* genus (Fig. 2). The fact that the new species is part of the *Dunaliella* genus was further confirmed by the use of a species of the genus *Asteromonas* as the outgroup of the phylogenetic tree. *Asteromonas*, a genus of the Asteromonadaceae, is the closest phylogenetic sister family to the *Dunaliellaceae* in the Chlamydomonadales (Hepperle et al. 1998; Nakada et al. 2008).

The 18S rRNA phylogenetic analysis is consistent with a neighbour joining (NJ) tree obtained from the alignment of 16S rRNA chloroplast gene sequences of other *Dunaliella* species (Fig. 3). Interestingly, the three uncultured *Dunaliella* species more closely related to *Dunaliella atacamensis* are aquatic species found in the hypersaline Lake Tebenquiche (Demergasso et al. 2008), located about 285 km inland in the Atacama Desert. Alignment of the 16S rRNA sequences of these three uncultured species with that of *Dunaliella atacamensis* revealed an identity of 94%, strongly suggesting that the latter is indeed a different species. The same identity was obtained with the 18S rRNA sequences.

For the *psaB* gene, the maximum-likelihood tree (Fig. 4) demonstrated that the new *Dunaliella* species was recovered in a clade (with a 98% of bootstrap support) together with other homonymous species constituting a monophyletic group. The new *Dunalliela* species is a sister form to *D. salina* strain CCAP 19/18. The long branch of

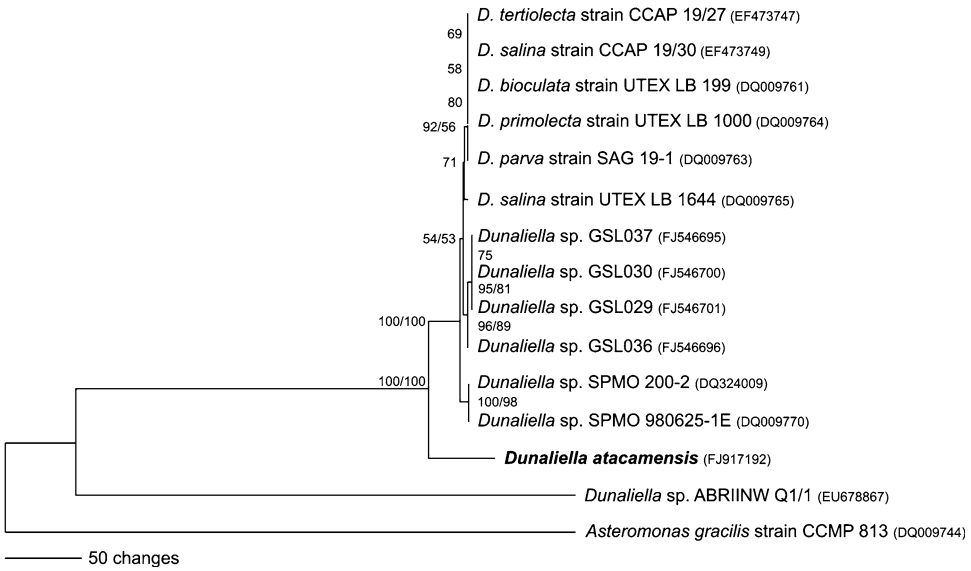

Fig. 2 Neighbour joining (NJ) and maximum parsimony (MP) strict consensus tree obtained from the aligned 18S rRNA *Dunaliella* gene sequences using PAUP. The maximum parsimony is a strict consensus of 75 trees. The *numbers* on the nodes represent bootstrap values with 1000 replicates for both NJ/MP

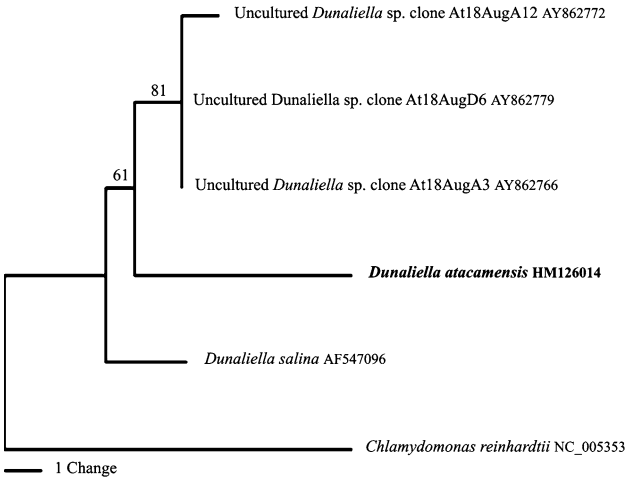

Fig. 3 Neighbour joining (NJ) tree obtained from the aligned 16S rRNA gene sequences of *Dunaliella* species using PAUP. *Numbers* close to the nodes represent 5000 replicates bootstrap values

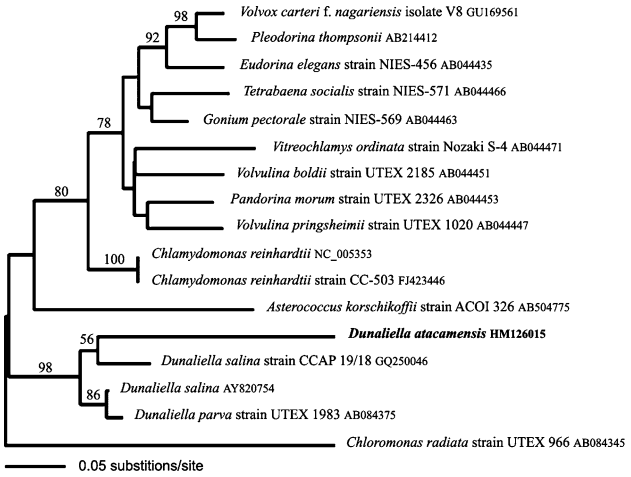

Fig. 4 Maximum likelihood (ML) tree obtained from the aligned *psaB* gene sequences of *Dunaliella* species using PAUP. *Numbers* close to the nodes represent 1000 replicates bootstrap values

Dunaliella atacamensis in relation to its sister species suggests that this new microalga is highly divergent with respect to other congeneric forms, consistent with the adaptations to its new subaerial habitat. Thus, different molecular markers, both nuclear (18S rRNA) and chloroplast encoded (16S rRNA and *psaB*), suggest that *Dunaliella atacamensis* is a valid species of the genus. On the other hand, *rbcL* specific primers amplified a 838-bp product corresponding to the ribulose-l,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit *rbcL* plastid gene. However, this gene resulted to be saturated and could not be used to reconstruct the phylogeny. Nevertheless, the analysis using the Megablast option for highly similar sequences of the BLASTN algorithm against the NCBI non-redundant database showed that most of the sequences producing significant alignments with maximum identity corresponded to *Dunaliella* species.

Bright field (BF), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and confocal laser scanning (CLSM) showed some of the characteristic internal ultrastructures described for *Dunaliella* species. SEM revealed groups of algal cells firmly attached to the silk threads (Fig. 5a). A well-defined pyrenoid surrounded by starch bodies and the thylakoid membranes of the chloroplast typical of *Dunaliella* cells were observed by TEM (Fig. 5b). CLSM micrographs confirmed the ultrastructural elements detected by TEM (pyrenoid and chloroplast). The functionality of the chloroplast in this habitat can be inferred by the strong autofluorescence of its associated chlorophyll (Fig. S2). A preliminary characterization of the photosynthetic pigments showed the typical combined absorption spectrum of chlorophyll *a* and carotenoid pigments previously reported for *Dunaliella* species (Fig. S3) (Smith et al. 1990; Evangelista et al. 2007).

Adaptations evolved for a subaerial habitat

As opposed to the remaining aquatic members of this group of algae, which lack a rigid cell wall and have two flagella, this sub-aerially adapted *Dunaliella* exhibits a well-developed layer of EPS and does not possess flagella (Figs. 5, 6). We also stained our samples with red ruthenium, a water-soluble hexavalent polycation dye, long used to stain acidic pectins and oxidized cellulose, both being typical components of cell walls (Sterling 1970). Red ruthenium facilitates deposition of osmium, which increases the electron density of the anionic polysaccharides. As shown in Fig. 6, we obtained a positive red ruthenium staining (panels a and c) compared with the unstained control (panels b and d). Red ruthenium has also been used for staining bacterial glycocalyx, since it stains its polysaccharide components (Fassel and Edmiston 1999). In our case, further extension of the electron dense exopolysaccharide matrix was not observed compared with the controls, as typically seen in reported glycocalyx (Cagle et al. 1972). Thus, it can be inferred that in our case a more "cell wall type" structure is observed.

It is well known that *Dunaliella salina* strains produce exopolysaccharides (EPS) formed by glucose, galactose, fructose and xylose in response to the environmental stress caused by increasing salt concentrations (Mishra and Jha 2009). EPS have been shown to be involved in the efficient capture and retention of ambient water by cyanobacteria in extreme environments (Shaw et al. 2003; Or et al. 2007), suggesting a similar role for the cave-inhabiting *Dunaliella atacamensis*. Another conserved adaptation shown by the *Dunaliella* genus which could be

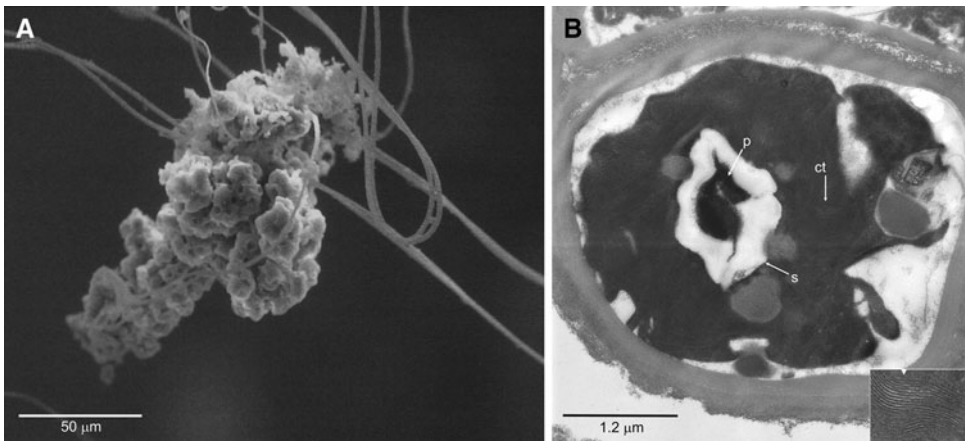

Fig. 5 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) micrographs of the cave-inhabiting subaerial *Dunaliella*. **a** SEM micrograph of group of cells attached to the silk threads. **b** TEM micrograph showing the internal structure of *Dunaliella atacamensis* cells; *p* pyrenoid, *s* starch bodies, *ct* chloroplast thylakoids. The *inset* shows a detail of the chloroplast tylakoids

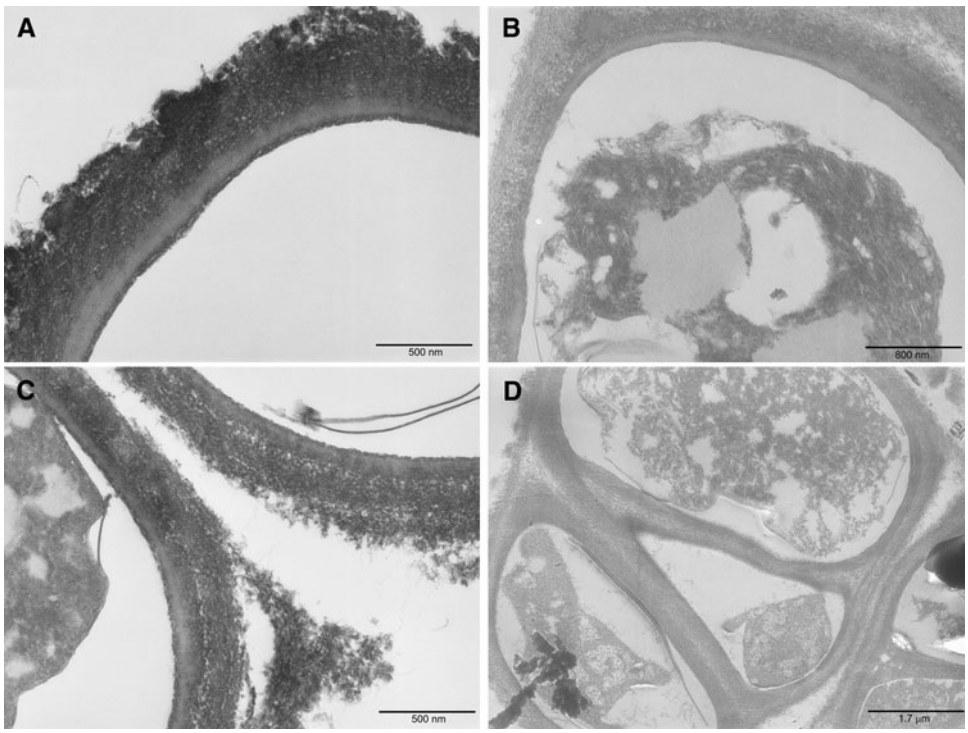

Fig. 6 Red ruthenium staining of cave inhabiting *Dunaliella* extracellular polymeric substances. **a** and **c** red ruthenium stained samples. **b** and **d** unstained controls

advantageous for internal water retention in a subaerial habitat is the production of the compatible solute glycerol in response to dehydration or extracellular osmotic pressure (Benoit et al. 2007; Chen and Jiang 2009). When subjected to a hyperosmotic shock (salt stress), *Dunaliella* cells rapidly shrink and synthesize massive amounts of glycerol that increase the internal osmolarity until the cells resume their original volume (Kaçka and Dönmez 2008). Experiments to find out whether *Dunaliella atacamensis* accumulates glycerol under conditions of hydric stress are now under way.

In relation to the colonial growth pattern of the cave *Dunaliella*, a "palmella" stage formation has been reported under reduced salinity conditions (≤10% NaCl) for other *Dunaliella* species. This pattern has been observed with *D. salina* and *D. viridis* onto submerged hard substrates, both in culture and in situ (Brock 1975). In the palmella stage, the cells usually lose their flagella and eyespot, become more rounded and excrete a layer of EPS in which they repeatedly divide, thus forming colonies of green cells (Borowitzka and Silva 2007). These morphological characteristics are strikingly similar to the ones observed in the colonies covering the spiderwebs (Fig. 1b). Massyuk (1973) described one variety of *Dunaliella viridis* where the palmelloid stage seemed to be the dominant stage of its life cycle. Strain MUR203 (=CCAP19/34) studied by Borowitzka and Silva (2007) also shows the palmella stage as the dominant form in culture conditions.

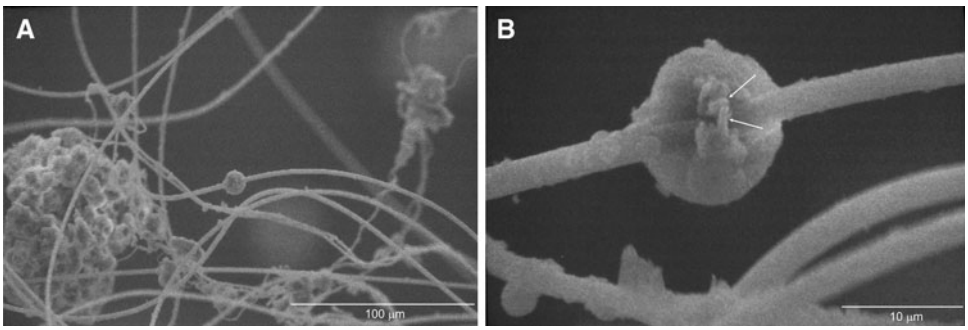

Fig. 7 Cave inhabiting *Dunaliella* flagella-like structures. **a** SEM micrograph of colonies and a single *Dunaliella* cell attached to the spider web threads. **b** Detail of single cell attachment shown on **a**. *White arrows* point to the short stub flagella-like structures

Intriguingly, in one of the few SEM micrographs illustrating single cells, small stub-like structures reminiscent of flagella were observed as point of contacts attaching the cell to the spiderweb thread (Fig. 7). These stub-like structures are comparable to short flagella mutants of *Dunaliella salina* previously described (Vismara et al. 2004) and could have the function of primary "clinging" devices.

On the other hand, as shown by the well-known equation of photosynthesis, $6CO_2 + 12H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O$, the availability of water and CO_2 may explain some of the most distinctive adaptations of the cave *Dunaliella*. This alga can only use CO_2 and bicarbonate as inorganic carbon sources (Hosseini Tafreshi and Shariati 2009). Since the diffusion of CO_2 is low in aquatic environments, green algae use the pyrenoid as a subcellular structure that colocalizes a CO_2 concentrating mechanism and the RuBisCo enzyme, thus maximizing CO_2 fixation (Kaplan and Reinhold 1999). Hence, the prevalence of a pyrenoid structure in cells adapted to a subaerial environment where CO_2 is not expected to be a limiting factor may reflect an adaptation previously useful at its former aquatic habitat. Alternatively, the preservation of a pyrenoid might be advantageous in a subaerial habitat since the thick layer of EPS now covering the cells could lower CO_2 diffusion and could be an advantage for carbon fixation in this extreme environment.

In summary, different features previously evolved by *Dunaliella* species for living in hypersaline environments result highly advantageous for adapting to a subaerial environment with reduced water availability and perhaps different light regimes than those experienced by its aquatic ancestors (i.e., colonial growth, pigments for avoiding photodamage, salt tolerance, glycerol production, EPS production). This process recapitulates the adaptations evolved by green algae for the sea-to-land transition (Karol et al. 2001; Lewis 2002; Chapman et al. 2002; Delwiche et al. 2002; McCourt et al. 2004). The transition from aquatic algae to land plants was undoubtedly one of the major events in the history of life and all evidence indicates that this transition was not the result of a single 'key innovation'. Instead, it appears to have arisen from a group of emergent properties resulting from complex interactions between different adaptations. As proposed by Delwiche et al. (2002) and Lewis (2002), as algae moved to land they could make an efficient use of resources by exploiting a range of previously evolved traits that are likely to have played a key role in this successful process of extending the habitability range. These include cell wall biochemistry, desiccation resistance and tolerance, structural complexity, as well as various reproductive strategies some of which are shown by the subaerial *Dunaliella* species. As previously noted by González et al. (2009) *Dunaliella* species show an enormous physiological variability, and our findings represent a beautiful example of this assertion.

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