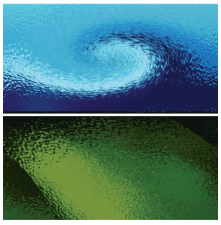
Transitional Waters Bulletin
TWB, Transit. Waters Bull. 4 (2010), n. 2, 60-70
ISSN 1825-229X, DOI 10.1285/i1825229Xv4n2p60
http://siba-ese.unisalento.it



RESEARCH ARTICLE

Morphological and molecular identification of a strain of the unicellular green alga *Dunaliella* sp. isolated from Tarquinia Salterns

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Abstract

1 - Algae of the genus *Dunaliella* are among the most studied micro-algae. They are used for the production of feed, for nutritional reinforcement as a vitamin A precursor and for pharmaceuticals and fine chemicals.

2 - The current taxonomy of the genus is based on morphological and physiological attributes including the ability of some species to grow over wide salinity ranges and at extreme salinities, as well as the accumulation of high levels of *β*-carotene. The taxonomic status of the genus *Dunaliella* involves some uncertainty, moreover it is very difficult to compare results from different authors, owing to uncertainty on names and species.

3 - In this work, we compare morphological and molecular analysis to characterize a strain of *Dunaliella* isolated from Tarquinia salt ponds. Samples of natural populations of the unicellular green alga, were collected at various times during the study period to detail the vegetative motile cells and the different stages of its life cycle microscopically. The ITS1 and ITS2 regions were used for the molecular identification analysis. Conserved oligonucleotides of 18S rDNAs (MA3) and species-specific primers (DSs), designed from variable sequences, were used to corroborate the identification.

4 - Blast results indicated that our sequences matched at the 100% level with *Dunaliella salina* Teod reported in Gen Bank. Consequently, based on comparative cell morphology and molecular analysis, the new *Dunaliella* isolate from Tarquinia salt ponds was classified as *D. salina*.

Keywords: *Dunaliella*, internal transcribed spacer, 18S rDNAs, PCR, taxonomy.

Introduction

Among the algae, *Dunaliella* is the only eukaryotic and photosynthetic organism able to grow in media containing concentrations of salt between 0.1M and saturation (approximately 5.5 M). This unusual performance comes from the ability of these algae to osmoregulate by producing and accumulating intracellular glycerol in response to the external salt concentration. Natural habitats of *Dunaliella* include brine lakes, oceans or salt marshes, but were water bodies contain more than 15% salt there is essentially a unialgal suspension of *Dunaliella*. The high salinity requirements of *Dunaliella*, minimise the number of competitors and predators, and allow it to be considered the most successful microalga in mass cultivation. Most studies of the genus have focused on physiological and biochemical questions. The main interest in this research project stems from the potential of these microalgae as a source of pigments, in particular *β*-carotenene, accumulated by some species within electrodense oily globules in the inter-thylakoid spaces of the chloroplast (Lorenz and Cysewski, 2000; Ip *et al.*, 2003). The

presence of 9-*cis*-*β*-carotene and all-*trans*-*β*-carotene isomers has been identified and confirmed in globules localised peripherally very close to the plasma membrane of *D. salina* and *D. bardawil* (Ben-Amotz *et al.*, 1982; Raja *et al.*, 2004). The importance of *Dunaliella*-derived *β*-carotene pigments is that they have different applications as a yellow colorant for products such as noodles, confectionery, beverages and health foods, and in the cosmetic and pharmaceutical industries as a colorant, as an antioxidant, as a vitamin A precursor, and as an anti-cancer agent (Borowitzka, 1995; Chidambara *et al.*, 2005; Levi *et al.*, 2000; Raja *et al.*, 2007; Hemaiswarya and Doble, 2006;). These pigments can also play an important role in the immune response (Hughes *et al.*, 1997), in neoplastic transformation, in the control of growth and in intracellular communication (Sies and Stahl, 2005). Ben-Almotz *et al.* (1982) have indicated that *D. salina* and *D. bardawil*, a close relative, possibly a subspecies of *D. salina* (Borowitzka and Siva, 2007; Gonzàlez *et al.*, 2001) are the only known *β*-carotene hyperproducer species of the genus (up to 10% of the dry algal biomass), when exposed to specific environmental conditions. In this contexst, isolation and identification of strains from natural habitats and subsequent evaluation of physiological attributes are important to obtain hyper-productive strain. These strains can be used for further development of biotechnology in the mass cultivation of these species. It is necessary to investigate alternative strategies for the high production of biomass and of enriched carotenoids. First detected in 1838 in saltern evaporation ponds in the south of France by Michel Felix Dunal (Dunal, 1838), the organism was named after its discoverer by Teodoresco in 1905. Since Teodoresco's description of *Dunaliella*, many species have been described from a wide variety of habitats (Borowitzka and Borowitzka, 1988). In his revision of the genus, Massyuk (1973) recognised 29 species. However, a controversy still exists regarding the identification of some species within the genus *Dunaliella*. It is therefore important to distinguish unambiguously whether some putative species actually represent the same species. A combined molecular, morphological and physiological approach, is important for evaluating the current classification. The most recent data indicate that molecular identification provides a useful tool to distinguish between inter- and intra-specific morphologically similar species and mixed populations (Gòmez and Gonzàlez, 2004; Olmos *et al.*, 2000). Analysis of the ITS-1 and ITS-2 sequences is useful at the population and species taxonomic levels (Gonzàlez *et al.*, 2001). Species-specific oligonucleotides could be used to identify species from culture collections or from natural environments. In *Dunaliella* species, slight phylogenetic and taxonomic differences can conceal profound differences in the potential for production of metabolites such as carotenoids. Previous research has demonstrated an important correlation between the carotenogenic capacity of *Dunaliella* strains and polymorphisms at the level of the genome (Gòmez and Gonzàlez, 2001). It is therefore important to recognise that no unique set of conditions stimulate carotenogenesis in these microalgae. Rather, their ability to perform carotenogenesis is likely the consequence of an intrinsic capacity of each strain to respond to inductive factors. Thus, the first objective of the present study was to isolate and identify a strain of *Dunaliella* from Tarquinia salt ponds using morphological and molecular integrated approaches.

Materials and methods

Isolation of strains and growth conditions

Dunaliella was isolated from Tarquinia salterns, an artificial aquatic ecosystem consisting of 35 pools with different salinity,

located on the central Tyrrhenian coast, near Rome. Actually the area is protected by a State Reserve patrolled by the National Forest Corp. The study was performed during the spring and winter seasons (2008-2009), in different ponds at greater salinities, some showing reddish water do to their high microalgal concentrations. The samples were collected at various times during the study period to detail the vegetative motile cells microscopically and to define the different stages of the alga's life cycle. Water samples were collected in sterile bottles and transferred aseptically to the laboratory. Possible contaminants were eliminated by treating the samples with 2000 ppm of streptomycin sulphate for 30 min and then transferring the algae to antibiotic free medium. Stock cultures were maintained in 1 L conical flasks containing seawater enriched with f/2 Guillard's medium. The NaCl concentration of the medium was adjusted to a level of 22% w/v. The culture was maintained under controlled laboratory conditions at temperature of ca. 25±3°C, using illumination from cool white fluorescent lamps (40 µmol photon $m^{-2}s^{-1}$), slow shaking and a photoperiod of 12:12. The medium was inoculated with salinity acclimated cultures. Growth was monitored daily. Two strains, differentiated according to cell color, were maintained separately for the analysis and designed "red" (DR2) and "green" (DV6).

DNA extraction

Unialgal cultures of two strains of *Dunaliella* (DR2 and DV6) were analysed. DNA extraction was performed when the cells in liquid culture were in the exponential growth state. One ml each of the green (DV6) and red (DR2) algae cultures were centrifuged anymore for 2 min at 1000 rpm. The supernatant was discarded. The DNA extraction was performed with the CTAB method of Murray and Thompson (1980) modified.

PCR amplification, sequencing and phylogenetic analysis of sequences

The ITS1 (TW81 5'-GGGATCCTTTCCG TAGGTGAACCTGC-3') and ITS2 (AB28 5'-GGGATCCATATGCTTAAGTTCAGC GGGT3') primers were used to amplify the ITS region in the forward and reverse directions, respectively (Gòmez and Gonzalez, 2004). To amplify the 18S rDNA gene, the specific forward primer DSs (5'-GCAGGAGAGCTAATAGGA-3') and the conserved reverse primer MA3 (5'-GGAA TTCCGGAAACCTTGTTACGAC-3') were used. PCR amplification was carried out in a DNA Thermal Cycler (Gene Amp PCR System 2400 - Perkin Elmer). The conditions for the ITS1/ITS2 primer pair were 5 min at 95°C, 35 cycles of 1 min at 90°C, 2 min at 50°C, 1 min at 72°C and a final elongation step of 10 min at 72°C (Gonzàlez *et al.*, 1999). The conditions for MA3 -DSs primers were, 5 min at 95°C, (initial denaturation), 35 cycles of 1 min at 95°C (denaturation), 1 min at 52°C (annealing) and 2 min at 72°C (extension) and a final elongation step of 10 min at 72°C, (Olmos *et al.*, 2000). All PCR amplifications were performed in 50 µl master mix solution containing 10 mM Tris HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgSO_4$, 200 µM dNTP, 0.4 µM of each primer, 1.0 u Taq polymerase (Promega) and 10 ng of total DNA. The molecular weights of PCR-amplified products were calculated and confirmed using gel documentation system. PCR products were directly sequenced by The Macrogen Co (Korea). All sequences were checked for similarity using a basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). ITS sequences showing the highest score and other ITS sequences representative of *D. salina* were retrieved from Gen Bank (Table 1) and aligned with CR2 and CV6 sequences using ClustalX software version

Table 1 - Strains of *Dunaliella salina* analysed in this study, with their origin and the Gen Bank accession number of each taxon.

***Dunaliella* strains**	**Gen Bank Accession number**	**Geographic origin**
Dunaliella salina	EF473741	Israel, north Sinai, salt pond near Bardawil lagoon
Dunaliella salina	EF473744	Russia, dirty salt lake
Dunaliella salina (Ds18S1)	FJ360756	unknown
Dunaliella salina (Ds18S3)	FJ360758	unknown
Dunaliella salina	EF473746	Australia, hut lagoon
Dunaliella salina	DQ116743	Israel
Dunaliella salina (CCP19/30)	EU932917	unknown
Dunaliella salina	HQ231412	India
Chlamydomonas reinhardtii	AB511842	

1.8 (Thompson *et al.*, 1997). A neighbour-joining tree was obtained using the software MEGA version 4. Evolutionary distances were computed using the Maximum Composite Likelihood model. For analysis, 1000 bootstrap replicates were performed to assess the statistical support for the tree. Phylogenetic studies included *Chlamydomonas reinhardii* (Gen Bank AB511842) as the outgroup.

Results and discussion

Morphological analysis

The morphology and behaviour of the cultures were examined under an optical microscope. Periodic checks were performed to monitor the status of the microorganism in respect to the stage of its life cycle. According to Borowitzka and Siva (2007) vegetative motile cells, particularly the red cells, are spherical, oval, ovoid or pear-shaped. Adult green cells range from 5.6-15.4 micron in length and 3.8-12.6 micron in width; red cells range from 12.6-15.4 micron in length and 11.2-15.4 micron in width. Their forms change to bilateral, dorsoventral or asymmetrical under extreme conditions. Fig. 1 shows the appearance of the vegetative motile cells. These cells are characterised by their biflagellate status, with a flagella approximately equal in length to the length of the cell. An eyespot is usually observed at the flagellar end in green cells, but an eyespot is difficult to distinguish in red cells. We have observed that some cells are more rounded, lack flagellum, and excrete a slime layer in which they divide repeatedly to form numerous green cells. This condition, named the “palmella stage”, was described in response to extreme conditions such as an increase in salinity. Zygospores were present (Fig. 1e). They appeared spherical, with

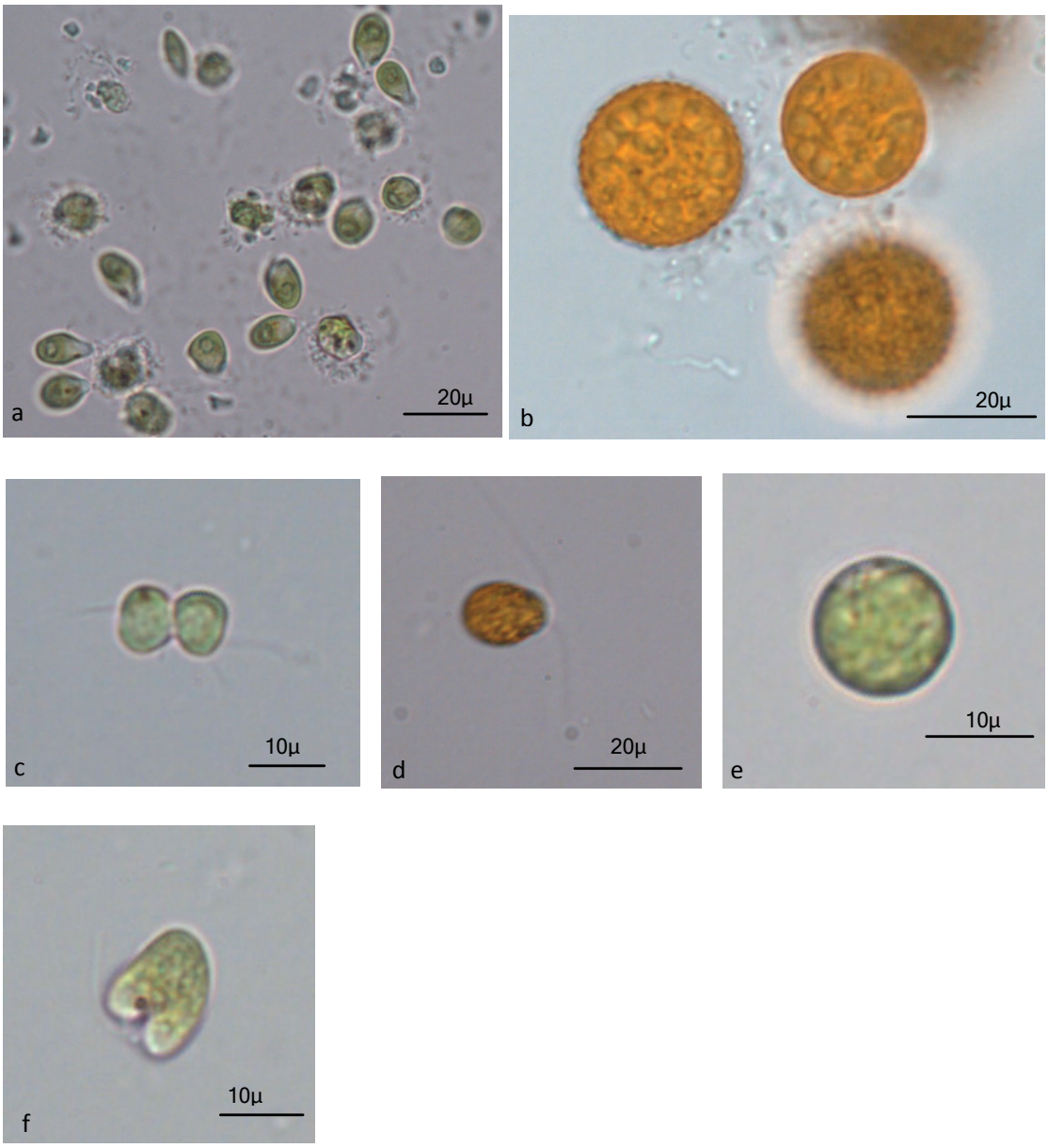

Figure 1 - Light micrographs of *Dunaliella salina*. Different stages of the life cycle: a) d) green and red cells (different shapes observed of flagellate status); c) f) aggregation of the green form; b) aplanospore with rough wall; e) zygospora stage.

smooth walls (17-19 micron in diameter), and with green to red contents. We also observed aplanospores, particularly in old cultures. These vegetative cysts are spherical, 12-20 micron in diameter, with an extremely resistant thick, rugose wall, and they often appear brownish to orange (Fig. 1b).

Molecular analysis

PCR amplification of the ITS region

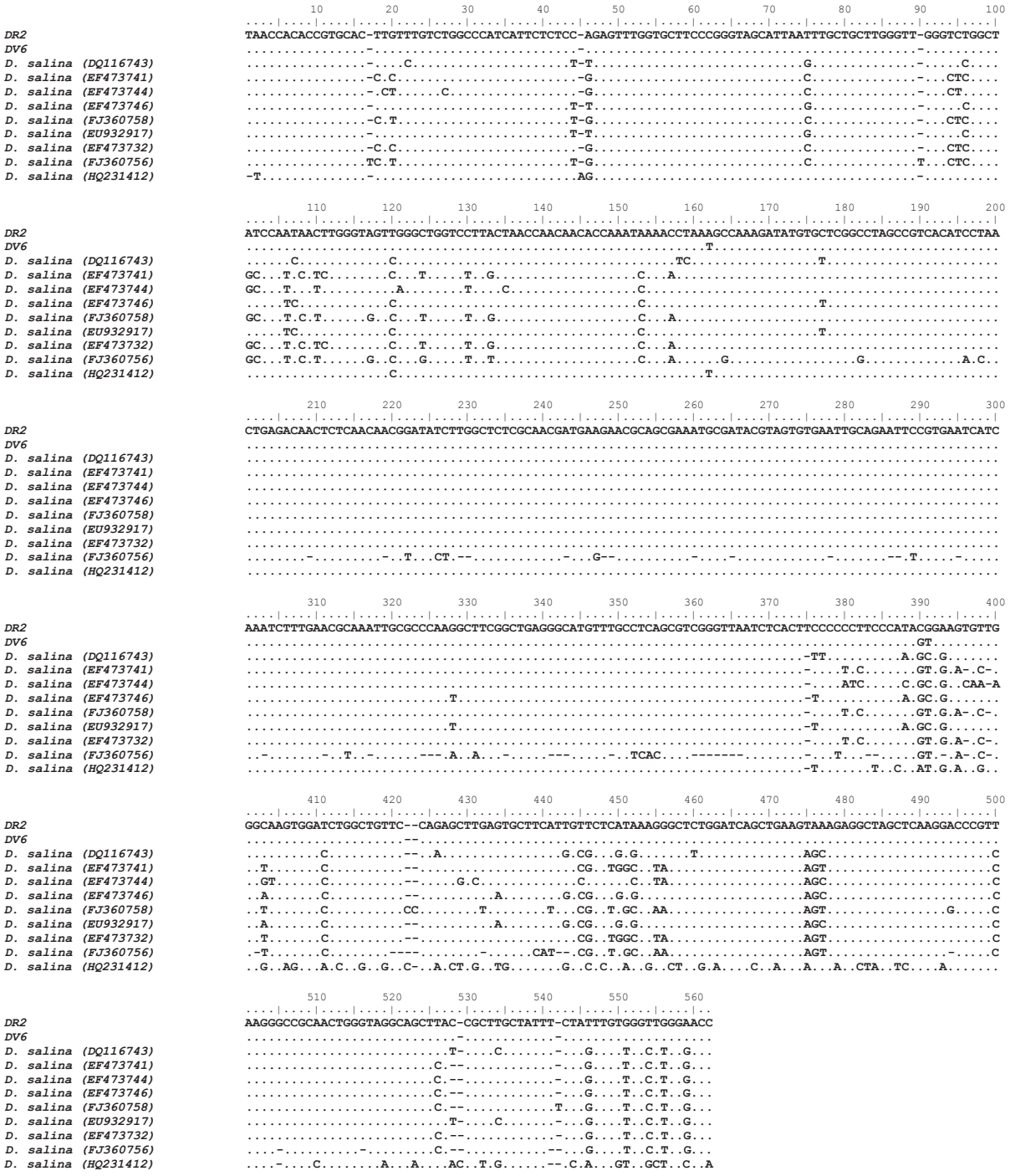

Figure 2. ITS (ITS1, 5.8rDNA and ITS2) partial sequence alignment of DR2 and DV6 *Dunaliella* strains with other *D. salina* species. Data for other strains were gathered from NCBI.

produced an amplicon of approximately 550 bp. Similarity search against the Gen Bank database using Blast showed that the sequences obtained are similar to the ITS sequences of *D. salina*. In particular, both strains, red (DR2) and green (DV6), showed

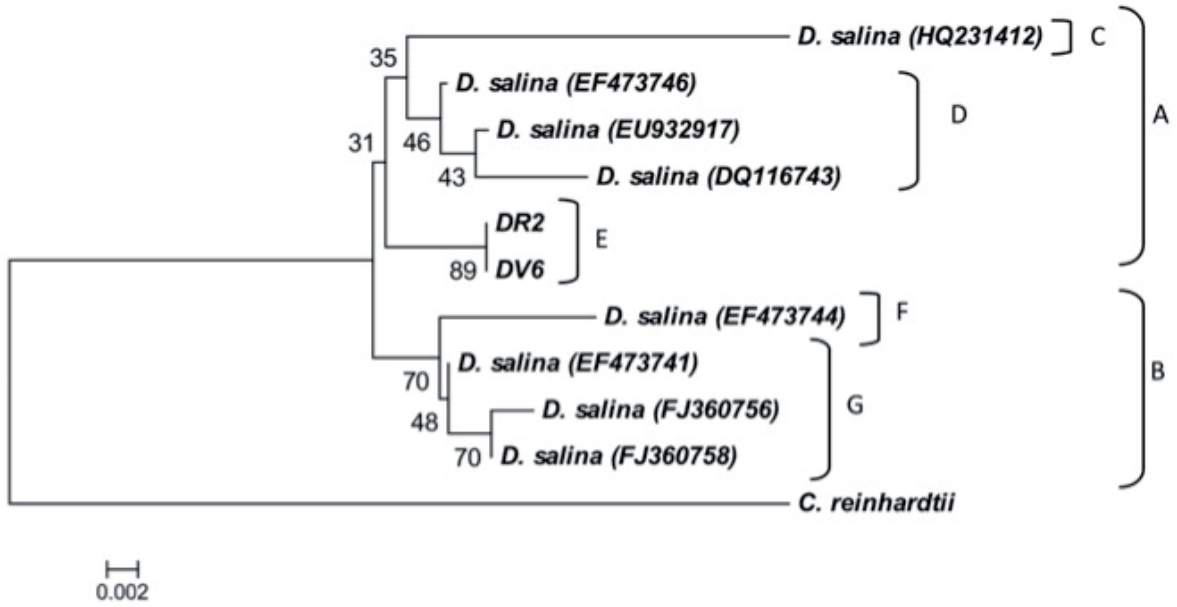

Figure 3. NJ bootstrap consensus tree showing the relationships among DR2, DV6, and *D. salina* species. Bootstrap values were calculated over 1000 replicates. *Chlamydomonas reinhardii* was considered as the outgroup (HQ231412).

high sequence similarity with *D. salina* strain CCAP 19/30 (accession number EU932917) and with *D. salina* strain Israel (accession number DQ116743), with 100% of query coverage and 92% of sequence identity (E-value 0.0). Fig. 2 shows the sequences DR2 and DV6 aligned with nine different strains, some of known geographic origin, whose ITS sequences were fully recorded at NCBI (Table 1). ITS nucleotide sequences for our strains exhibited similarities with other strains ranging from 82% to 94%. In order to evaluate the phylogenetic relationships between our isolate and other *D. salina* species in greater detail, ITS sequences for reference species were retrieved from Gen Bank and used to perform a phylogenetic analysis including *Chlamydomonas reinhardii* as the outgroup. The sequences considered in the phylogenetic analysis were selected on the basis of the Blast results and on the sequences cited in published works (Gonzalez *et al.*, 2001). Fig. 3 shows the corresponding dendrogram established by NJ. All sequences in the dendrogram were divided into two main clades (Fig. 3 A, B), showing bootstrap values 31 and 70, respectively. Within clade A, the isolates DR2 and DV6 appeared as a single group (subclade E) supported by a high bootstrap value (89) and differing from the strains included in subclades D and C. In the same clade (A), the isolate HQ231412 from India seemed to be genetically different from the other D. salina strains (EF473746, EU932917, DQ116743), even though the bootstrap value was low (35). The clade B included subclade F with the strain EF473744 from Russia, and subclade G, that was characterised by the presence of isolates EF473741(from Israel), FJ360756 and FJ360758 (unknown). To investigate the species level of our isolate, PCR amplification was carried out using variable and conserved sequences contained in the 18S rDNAs.

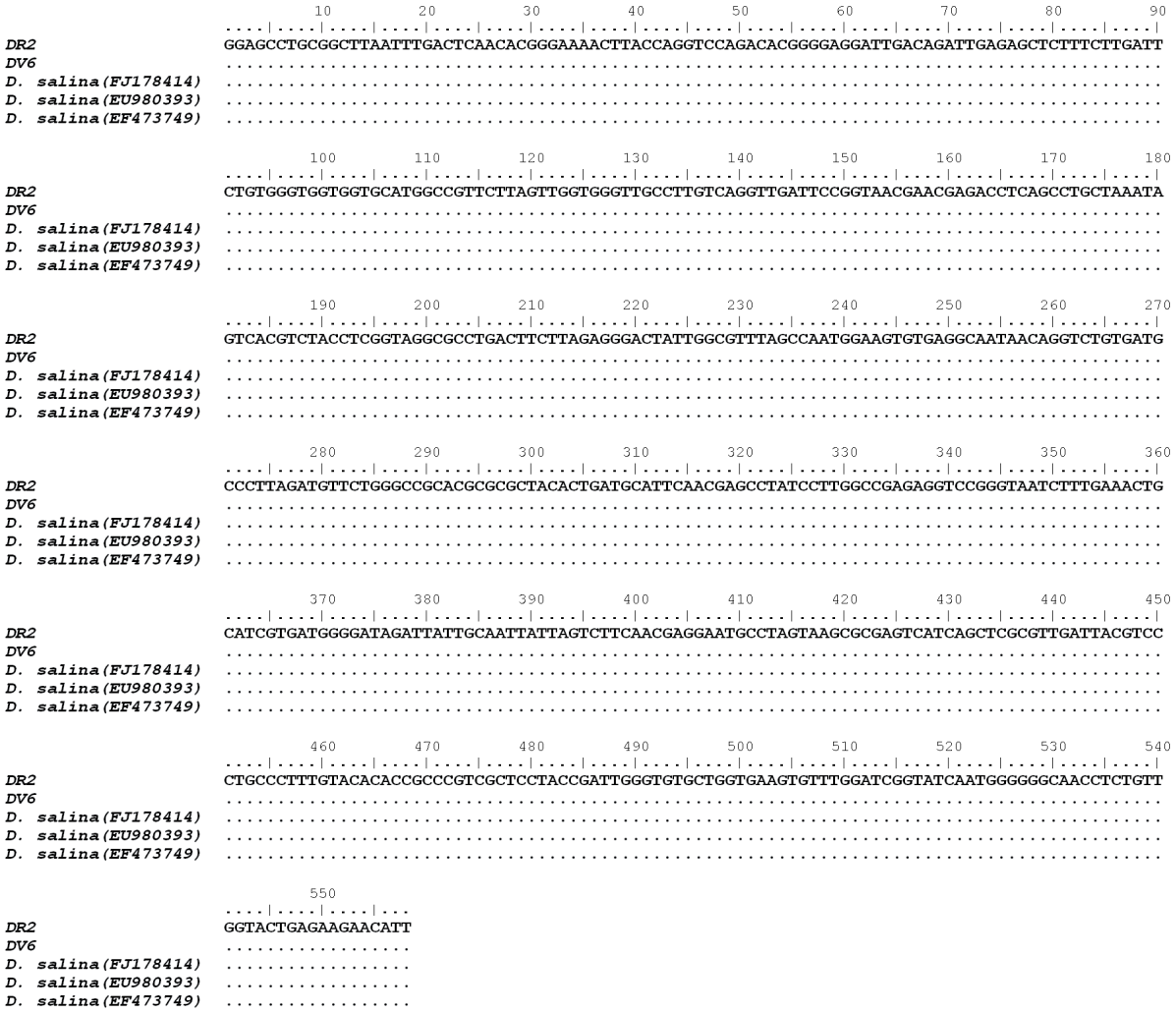

Figure 4. 18S rDNA intron alignment of DR2 and DV6 *Dunaliella* strains with sequences blasted in the Gen Bank database showing 100% sequence identity.

Species-specific primer DSs (Olmos *et al.*, 2000), designed from the first intron of the 18S rDNA of D. salina, was used in combination with MA3. The PCR product obtained from the samples CR2-CV6 that contained a single band of approximately 530 bp, was sequenced. The sequence data were aligned in the Gen Bank database. Blast results indicated that our sequences matched at a level of 100% with *D. salina*. The first three sequences that appeared at 100% of sequence identity (FJ178414, EU980393, EF473749) were aligned with our isolates (Fig. 4).

Conclusions

The use of *D. salina* species for the production of numerous biotechnological products is growing in importance. Morphological variability and the ability to adapt to changes in environmental conditions made puzzling the systematic of this genus, so that the correct identity of particular specimens is difficult to be determined. During the 19th century, Dunal's red flagellate algae were

observed by other biologists in salt lakes and hypersaline sites in Lorraine, France (Florentin, 1899), in Crimea (Butschinsky, 1897), Algeria (Blanchard, 1891), and Romania (Bujor, 1900). Different names were assigned to the organism by each investigator. The identification of the microorganisms has long been performed using morphological characteristics alone. Application of modern biotechnological PCR-based tools (molecular analysis) is considered an important approach to identification of microorganisms in natural populations. Concerning the genus *Dunaliella*, genetic investigations based on DNA studies started recently, with the goal of elucidating the phylogeny of the genus (Gonzàlez *et al.*, 1999, 2001). Internal transcribed spacer (ITS) analysis has revealed a great genetic similarity among some strains belonging to three different sections of the subgenus (*D. tertiolecta* from section Tertiolectae, *D. parva* from section *Dunaliella* and *D. peircei* from section Peirceinae). These similarities are greater than those found among strains within *D. salina*. Recent studies have confirmed that the 18S rDNA gene in *Dunaliella* contains a relatively conserved region of exons and a variable region of introns. Although the size of 18S rDNa is similar in *D. salina*, the position and the nucleotide structure of the introns differ. For these reasons, we considered important to perform molecular studies in tandem with morphological observations, as done in this work, since this is the only way to provide a clear association between morphological traits, genotypes and species name. The PCR-amplified and sequenced Internal Transcribed Sequence (TW81-AB28) in DR2 and DV6 showed a high sequence similarity with *D. salina* strain CCAP 19/30 (EU932917) and with *D. salina* strain Israel (DQ116743), with 100% of query coverage and 92% of sequence identity in both (E-value 0.0). The analysis of phylogenetic relationships between our isolates and other *D. salina* species showed two different evolutionary lineages (Clade A, B). Within clade A, our strains group is a distinct, well supported subclade (Fig. 3). ITS sequences are useful for characterising strains at the species level. They are particularly useful for predicting genetic relatedness and for studying the phylogeny of the genus *Dunaliella*. Furthermore, the analysis of the ITS region sequence indicates that this region in our isolate shows similarity ranging from 82% to 94% with other *D. salina* species (Fig. 2). We also considered the 18S rDNA gene. This sequence information is important to explain the differentiation and identification of various *Dunialiella* strains. The PCR amplification of 18S rDNA of the alga with primer MA3 and species-specific primer DSs resulted an efficient amplification. The amplified products showed highest sequence similarity (100%) with *D. salina* in both isolates (DV6-DR2) (Fig. 4). Microscopic examination of the red and green *Dunialiella* strains isolated in this study express the morphological attributes on which the taxonomy of the genus is founded (Borowitzka and Siva, 2007). Biflagellate cells were approximately oval or pear-shaped. Green cells were 5.6-15.4 micron long and 3.8-12.6 micron wide. Red cells were 12.6-15.4 micron long and 11.2-15.4 micron wide. An eyespot was usually observed. Moreover, we described a palmella stage that was produced in response to extreme conditions, such as an increase in salinity. We also observed aplanospores having rugose walls and zygospores, green to red in colour, with smooth walls. Moreover, in a study carried out by Pasqualetti *et al.* (2010), this unicellular green alga, exhibited an high halophytism with a certain degree carotenogenic ability. The change in cell colour from green to orange or red under specific extreme environmental conditions or under suboptimal culture conditions probably reflect the carotenogenic ability of

the vegetative cells, as previously signalled by other authors (Massyuk, 1973; Preisig, 1992). In conclusion, based on comparative morphology and molecular analysis, we identified the strains DV6 and DR2 isolated from Tarquinia salterns as members of the same taxon, and we classified them as *D. salina*.

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