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Identification and Characterization of a New Strain of the Unicellular Green Alga *Dunaliella salina* (Teod.) from Korea

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The unicellular green alga *Dunaliella salina* is a halotolerant eukaryotic organism. Its halophytic properties provide an important advantage for open pond mass cultivation, since *D. salina* can be grown selectively. *D. salina* was originally described by E. C. Teodoresco in 1905. Since that time, numerous isolates of *D. salina* have been identified from hypersaline environments on different continents. The new *Dunaliella* strain used for this study was isolated from the salt farm area of the west coastal side of South Korea. Cells of the new strain were approximately oval- or pear-shaped (approximately 16–24 μm long and 10–15 μm wide), and contained one pyrenoid, cytoplasmatic granules, and no visible eyespot. Although levels of β-carotene per cell were relatively low in cells grown at salinities between 0.5 to 2.5 M NaCl, cells grown at 4.5 M NaCl contained about a ten-fold increase in cellular levels of β-carotene, which demonstrated that cells of the new Korean strain of *Dunaliella* can overaccumulate β-carotene in response to salt stress. Analysis of the ITS1 and ITS2 regions of the new Korean isolate showed that it is in the same clade as *D. salina*. Consequently, based on comparative cell morphology, biochemistry, and molecular phylogeny, the new *Dunaliella* isolate from South Korea was classified as *D. salina* KCTC10654BP.

Keywords: Green alga, *Dunaliella salina*, β-carotene

Unicellular green algae of the genus *Dunaliella* are divided into two subgenera: *Pascheria* and *Dunaliella* [26, 27, 31]. The subgenus *Dunaliella* contains species that are remarkably halotolerant; that is, their growth range includes NaCl concentrations that are lower than seawater, <0.5 M, or reach saturation levels of >5 M [23]. According to the Morphological Species Concept, the type species *Dunaliella salina* Teod is defined as having a cell size between 16–24 μm long and 9.5–13.3 μm wide, with cells having one diffuse eyespot that is hardly visible by light microscopy [21, 23, 24, 32]. Unfortunately, morphological characteristics of cell size and form, number and position of eyespots, number and position of refractive granules, and the size and shape of the pyrenoid depend largely on the age of a culture and the growth conditions [2, 5, 6, 22]. Therefore, identification of new *Dunaliella* isolates is often difficult and the physiological as well as biochemical characteristics of the algae should be used to identify strains of the species *D. salina*. For example, strains of *D. salina* grow optimally at salinities between 6–12% [24, 29, 30], which corresponds to about 1–2 M NaCl. Moreover, cells of *D. salina* are green under optimal growth conditions, but they are orange when exposed to environmental stresses such as high salinity owing to the accumulation of β-carotene [14, 21, 24, 25, 31, 32].

Recently, the Phylogenetic Species Concept was used to classify the genus *Dunaliella* by molecular analysis of the nuclear rDNA internal transcribed spacer regions ITS1 and ITS2 [8, 10, 11, 19, 20], and also with analysis by Random Amplified Polymorphic DNA [17, 19, 20]. Specifically, analysis of the ITS regions for phylogenetic classification is very well suited to provide information about the species level and there is abundant information available [1, 7, 9, 14].

Species of *Dunaliella* are well known in the alga biotechnological industry and are employed widely, for example, for the production of valuable biochemicals, such as carotenoids. Some strains of *Dunaliella* such as *D. salina* and *D. bardawil* Avron et Ben-Amotz are cultivated commercially in large outdoor ponds and are harvested to produce dry algal meals, such as polyunsaturated fatty acids and oils for the health food industry, as well as

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coloring agents for the food and cosmetic industries [3, 4, 18, 22]. During the past decade, new isolates of *Dunaliella* sp. and the biochemistry of microalgae, along with the advances in biotechnology of microalgal mass cultivation, enabled this microalga to become a staple of commercial exploitation. In particular, the advent of molecular biology and mutagenesis in *Dunaliella* permitted enhancements in the carotenoid content of this green alga, making it more attractive for biotechnological applications.

This work was initiated to identify a new Korean isolate of *Dunaliella* using a combined phylogenetic, morphological, and physiological approach for classification. Based on the combined analyses, the new isolate was identified as belonging to the species *D. salina*. Currently, the new strain is being investigated further for possible use in biotechnology applications.

MATERIALS AND METHODS

Algal Strains and Growth Conditions

Dunaliella salina strain AC144 (CHL10) originates from Tunisia and was received from the Algobank Culture Collection in Caen, France. *Dunaliella salina* from Lake Tyrell in Australia was collected and kindly provided for this study by Dr. Dyall-Smith. *Dunaliella salina* KCTC10654BP, which was isolated from the salt farm area of the west coastal side of South Korea, was provided by Dr. Noh from the Korean Ocean Research Development Institute.

All strains were cultivated in artificial saline modified medium [31] with final concentrations of 40 mM Tris-HCl (pH 7.5), 5 mM KNO_3, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 8 μM $FeCl_3$, and 80 μM EDTA at pH 7.5. The micronutrients were at final concentrations of 150 μM H_3BO_3, 10 μM $MnCl_2$, 0.8 μM $ZnCl_2$, 0.3 μM $CuCl_2$, 2 μM Na_2MoO_4, 2 μM $NaVO_3$, and 0.2 μM $CoCl_2$ per 1 l medium. A sterile filtered bicarbonate solution (pH 7.5) was added to yield a final concentration of 25 mM.

Axenic cultures were grown in sterile 50-ml Falcon tubes on a shaker (150 rpm) under about 80 μmol photons m^{-2} s^{-1} of daylight provided by fluorescent lamps. The NaCl concentrations of the media were adjusted to levels of 0.5 M, 1.0 M, 1.5 M, 2.5 M, 3.0 M, and 4.5 M. Strains were routinely maintained on 1.5% agarose plates containing medium with 1.0 M NaCl under low light levels of <10 μmol photons m^{-2} s^{-1}.

Microscopy

For light microscopy, cells were grown until the early- or mid-log phase and pictures were taken using an inverted Motic AE31 microscope with an LWD PH40x/0.60 objective in combination with an Optem digital camera coupler (29-90-56, 25-70-15, and 25-70-14) and a Nikon Coolpix 4500 digital camera (Nikon Corp., Tokyo, Japan) with a zoom of 4× to obtain a maximal magnification of 400×.

DNA Extraction and Purification

For DNA isolation, cells were grown on 1.0 M NaCl plates containing 1.5% agarose. Cells from each strain were taken directly from these plates and DNA was isolated using the QIAGEN DNeasy Kit (QIAGEN Inc., Valencia, CA, U.S.A.) following the standard protocol.

PCR Amplification, Purification, and Sequencing

Standard PCR was performed using a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) and PROMEGA Master Mix #M7502 (Promega Corp., Madison, WI, U.S.A.) on a 100-μl mixture containing 200 ng of chromosomal DNA. For ITS1, the amplification was performed after 3 min of denaturation at 95°C using 36 cycles with one cycle consisting of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C. The ITS1 region was amplified using the primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (GCTGCGTTCTTCATCGATGC) (Bruns Laboratory, UC Berkeley, CA, U.S.A.) at a concentration of 10 pmol each. The ITS2 amplification was performed under similar conditions to those for ITS1, with the exception that the reannealing temperature was 55°C. The ITS2 region was amplified using the primer pair ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) (Bruns Laboratory, UC Berkeley, CA, U.S.A.) at a concentration of 10 pmol each. The resulting PCR products were purified using the QIAGEN MinElute PCR Purification Kit (QIAGEN Inc., Valencia, CA, U.S.A.). Purified products were verified on a 1.5% agarose gel and the purified ITS was sent for sequencing at the Rockefeller Sequencing Center (Rockefeller University, New York, U.S.A.). Routinely, both strands of the ITS regions were sequenced in two to four separate reactions with the primers used in the original PCR. The new sequences for strains from this work were deposited in the NCBI database and the accession numbers are listed in Table 2.

Sequence Alignment

The ITS1 and ITS2 sequences obtained for *Dunaliella* sp. from Korea, *Dunaliella salina* AC144, and *Dunaliella* sp. from Australia were edited with the program EditSeq (DNASTAR, Inc.) and aligned together with all other *Dunaliella* sequences using the program MegAlign (DNASTAR, Inc.). The combined data matrix (ITS1 and ITS2) consisted of 458 aligned basepairs (218 bp from ITS1 and 240 bp from ITS2) and 28 taxa. There were 109 (24%) phylogenetically informative sites. ITS1 sequences were missing for five taxa, and ITS2 for one taxon.

Phylogenetic Analysis

A parsimony analysis was conducted using the software WinClada [25] and NONA [13] with the following options selected: unconstrained search, a maximum of 10,000 trees saved (hold), 100 search replicates with random addition (mult* N), three starting trees in each replicate (hold), all most-parsimonious trees saved, branch swapping set to multiple TBR+TBR (mult*max*), and the random starting seed set to 0. Missing data were treated as uncertainties ("?"). As an outgroup, the taxon "*D. salina*" from Vietnam (NCBI# AJ496573) was used, since phylogenetic analysis showed that it was a basal group and as such not part of the *Dunaliella salina* species complex. The strict consensus tree was calculated using WinClada, as well as tree length and homoplasy indices such as the consistency index and retention index (ri) [12]. Branch support analysis was performed with WinClada, and jackknife analysis [13] was performed using the following settings: 200 replicates, 10 search replicates/replicate, one starting tree, don't do max*, and save consensus.

Pigment Analysis

For HPLC analysis, an HPLC Hewlett Packard Series model 1100 equipped with a Waters Spherisorb S5 ODS2 4.6×250 mm Cartridge Column was used. Two ml of algal suspension was centrifuged in an Eppendorf centrifuge at 14,000 rpm for 2 min. The pigments were extracted from the algal cells upon addition of the 200 µl filtered 90% acetone to the pellet, followed by vortexing at maximum speed for 1 min. The extract was centrifuged in an Eppendorf centrifuge at 14,000 rpm, and 15 µl of the filtered supernatant (0.2 µm nylon filter) was subjected to HPLC analysis. The pigments were separated using a solvent mixture of acetonitrile, ddH_2O, triethylamine, and ethyl acetate. During the run, the solvent concentrations were 90% acetonitrile, 10% water, and 0.01% triethylamine from time 0 to 1 min. From time 2 min to 14 min, the solvent mixture consisted of 86% acetonitrile, 9.6% water, 0.01% triethylamine, and 5% ethyl acetate. From time 15 min to 21 min, 100% ethyl acetate was used. A post-run followed for 9 min with the initial solvent mixture. The flow rate was maintained at 1.0 ml/min throughout the run. Pigments were detected at a wavelength of 445 nm with a reference wavelength of 550 nm. Concentrations of individual pigments were determined from the HPLC profiles calibrated with standard samples of chlorophyll *a* and β-carotene.

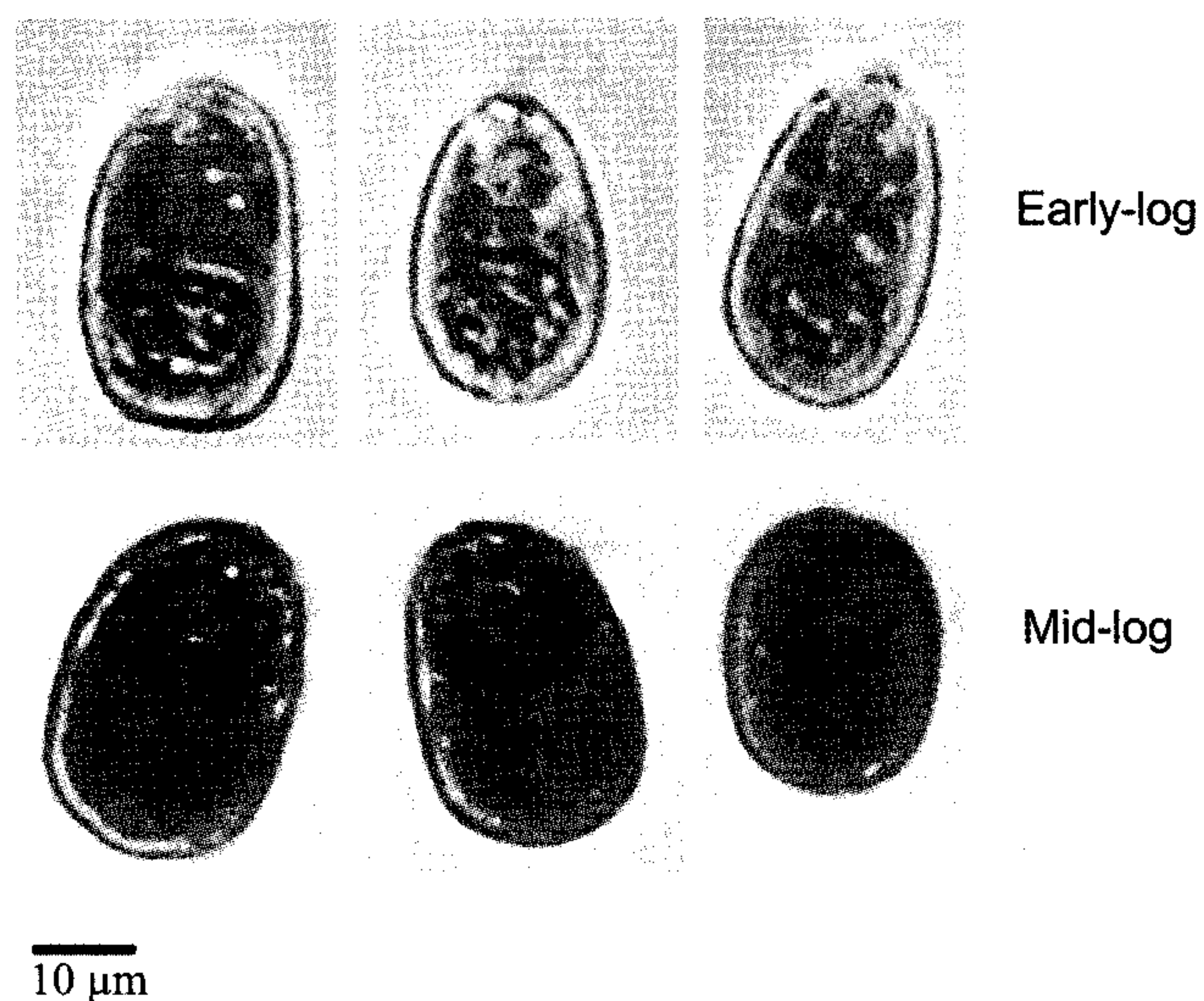

Fig. 1. Comparison of cells of a new Korean isolate of *Dunaliella* grown at a salinity of 1.5 M NaCl.
Note that cells in the early-log phase of growth have a different morphology than cells from the mid-log phase. The microscopic magnification is 400× with an additional image zoom of 200%.

RESULTS AND DISCUSSION

The species *D. salina* has been reported to exist in hypersaline environments on almost all continents [15]. Table 1 shows a comparison of various *D. salina* strains isolated from several different continents. This study focused on one new *Dunaliella* isolate from South Korea. Morphological, biochemical, and molecular characteristics were used to identify this strain as belonging to the species *D. salina* (TEOD).

Morphological Analysis

According to Teodoresco [32] and Hamburger [21], vegetative motile cells of *D. salina* are ellipsoid, cylindrical to pear-shaped cells of various sizes with adult cells in the range of 16–24 µm long and 9.5–13.3 µm wide. Previous reports noted that cell morphology depends on the age of the culture. Therefore, for morphological analysis of the new isolate from South Korea, cells of the new isolate were first grown in batch culture, and cell morphology was compared depending on the age of the culture. Fig. 1 shows

Table 1. Comparison of different *D. salina* isolates that originate from various locations, covering several continents.

Species/Isolate	From	Source	Reference
D. salina CCAP19/18	Hutt Lagoon, Australia	NA	NCBI
D. salina	Tanggu, China	NA	NCBI
D. salina	Yucatan, Mexico	NA	NCBI
D. salina CONC-001	Chile	NA	NCBI
D. salina CONC-006	Salar de Atacama, Chile	NA	[20]
D. salina CONC-007	Salar de Atacama, Chile	NA	[20]
D. salina UTEX LB1644	Baja California, Mexico	NA	NCBI
*D. salina SAG*184.80	Unknown	SAG, Germany	This study
D. salina AC144 (CHL10)	Tunisia, North Africa	Algobank, France	This study
D. salina DCCBC1	Lake Tyrell, Australia	Dr. M. Dyall-Smith, Australia	This study
D. salina DCCBC27	Salterns, Geelong, Australia	Dr. M. Dyall-Smith, Australia	This study
D. salina KCTC10654BP	West Coast, South Korea	Dr. J. H. Noh	This study
D. bardawil UTEX LB2538=ATCC 30861	Near Bardawil Lagoon, North Sinai, Israel	UTEX Culture Collection, USA	NCBI
D. sp. CCAP19/12	North Sinai, Israel	CCAP Culture Collection, UK	This study
D. salina UTEX200	Dirty salt lake, Russia	NA	NCBI
D. salina sp. 006 Coleman and Brown	Unknown	NA	[10]
D. (salina) sp.	Unknown	NA	NCBI

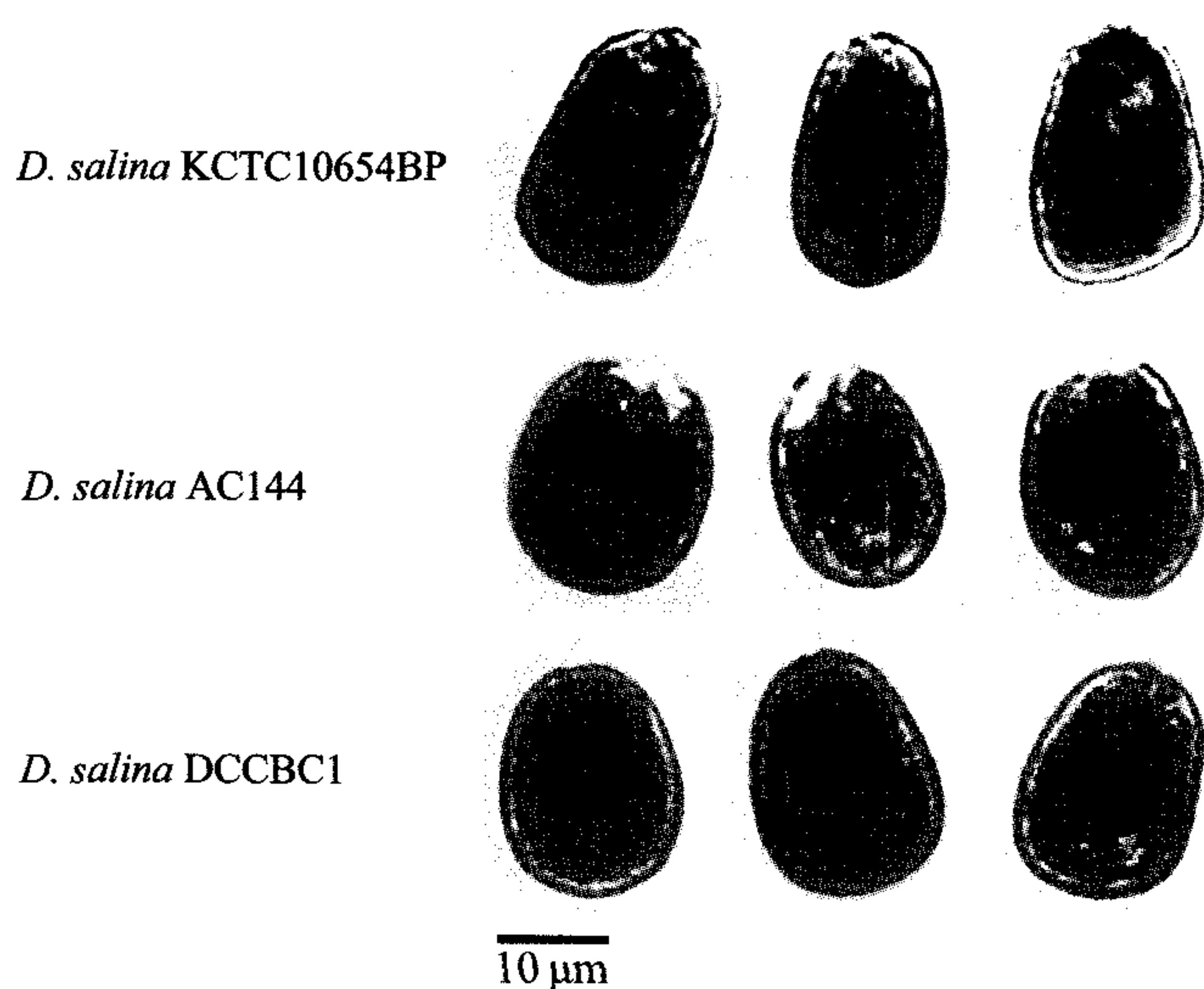

Fig. 2. Typical cells of the new Korean *Dunaliella* isolate, *D. salina* AC144, and *D. salina* DCCBC1 grown on a shaker in 1.0 M NaCl medium under ~80 μmol photons m^{-2} s^{-1}.
The microscopic magnification is 400× with an additional image zoom of 200%. Note that *D. salina* AC144 and *D. salina* DCCBC1 are morphologically virtually identical although they belong to two different phylogenetic clades. In contrast, cells of the new Korean *Dunaliella* isolate are greener and the cytoplasmic vacuoles are brighter than those of the other two *D. salina* strains.

photographs of cells revealing heterogeneity in morphology, depending on the growth phase of the culture. Consequently, only cells from the light-limited growth phase were used for comparative analysis of different strains.

Next, cells of the new isolate were compared with cells of the strains *D. salina* AC144 and *D. salina* DCCBC1. Cells of all strains were cultivated under identical growth conditions. Fig. 2 shows three representative cells from the mid-log phase of batch cultures for each strain. In general, cells of all strains were approximately oval- or pear-shaped (approximately 16–24 μm long and 10–15 μm wide), and contained one pyrenoid, cytoplasmic granules, and no visible eyespot. Cells of the new isolate appeared morphologically highly similar to the species *D. salina* described by Teodoresco [32] in Tafel VIII, Figs. 1–3 of that paper. At 1.0 M NaCl, cells of *D. salina* Korea were on average 17.9 μm long and 12 μm wide. Therefore, the morphology, based on the cell size and the presence of a barely visible eyespot, met the criteria for inclusion in the species *D. salina*. However, as shown exemplarily in Fig. 2, one noticeable difference between strains was that cells of the *Dunaliella* isolate from South Korea were noticeably greener than those of other *D. salina* strains, a phenomenon observed independent of the salinity of the growth medium. Moreover, cells of *D. salina* AC144 and *D. salina* DCCBC1 contained cytoplasmic granules of a much darker color than cells of the new Korean *Dunaliella* isolate.

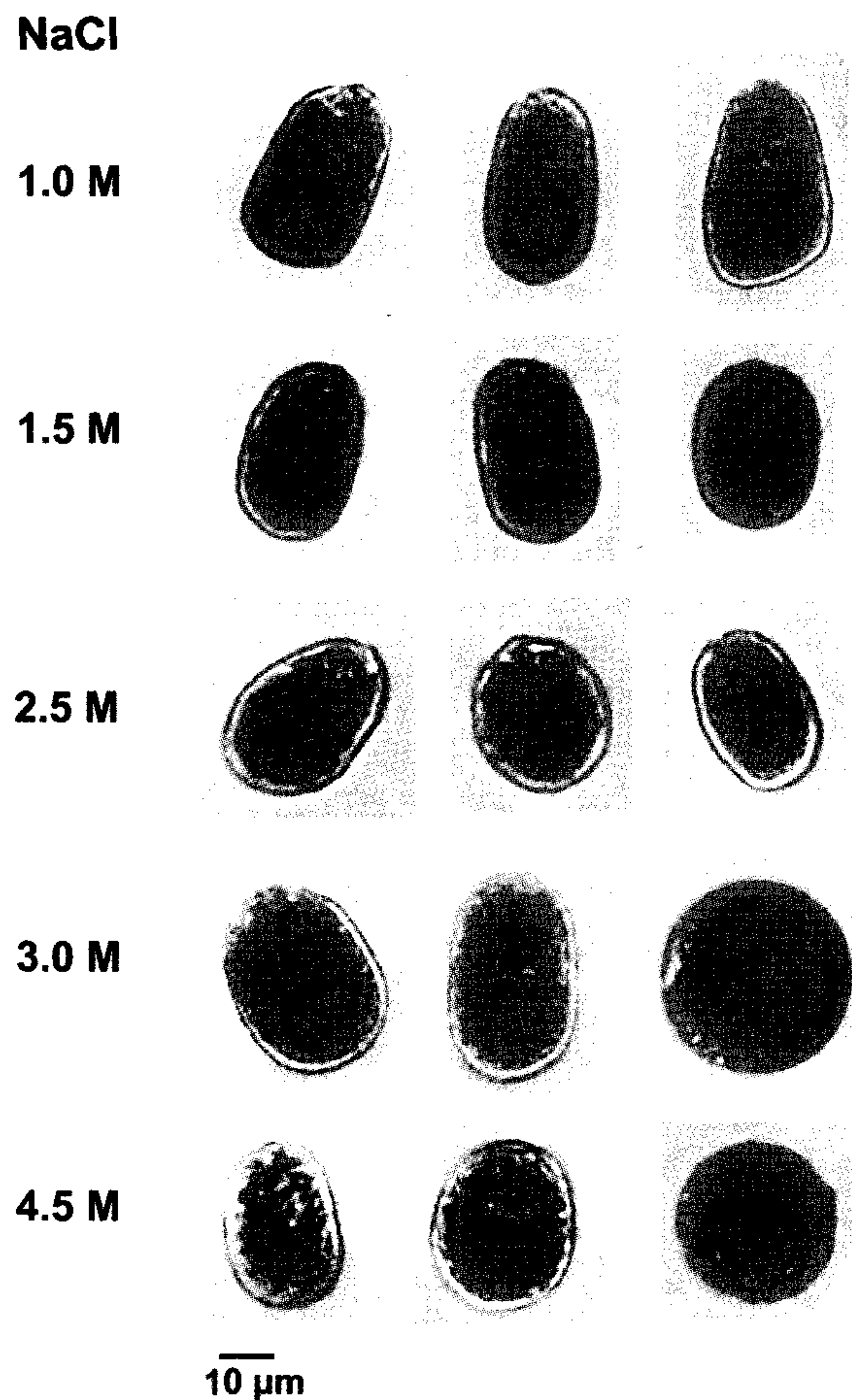

Fig. 3. Cells of a new Korean isolate of *Dunaliella* are shown being grown at different salinities (NaCl concentrations).
Note that the cells of this new isolate are turning orange in color when salt-stressed. Three different photos were taken from cells at each salt concentration.

Analysis of Secondary Carotenoid Accumulation

According to Teodoresco [32], cells of *D. salina* turn orange under stress because of an overaccumulation of β-carotene [25]. In the past, several new *Dunaliella* isolates were misidentified, since only cell morphology was considered. To test for overaccumulation in the new *Dunaliella* isolate, cells were cultivated under identical growth conditions in media containing various salinities ranging from 0.5 M to 4.5 M NaCl. Fig. 3 shows cells of the Korean isolate from the mid-log phase of growth. At an optimal salinity of about 1.5 M NaCl, cells were visibly green and cells grown at higher salinities only showed a slight color change towards orange. Surprisingly, cells did not turn bright orange, as known for cells of other *D. salina* strains, suggesting that cellular levels of β-carotene in the Korean strain were much lower than in the other strains. As overaccumulation of β-carotene is a biochemical characteristic for cells of the species *D. salina* [25, 31], this result implied that the new *Dunaliella* isolate from

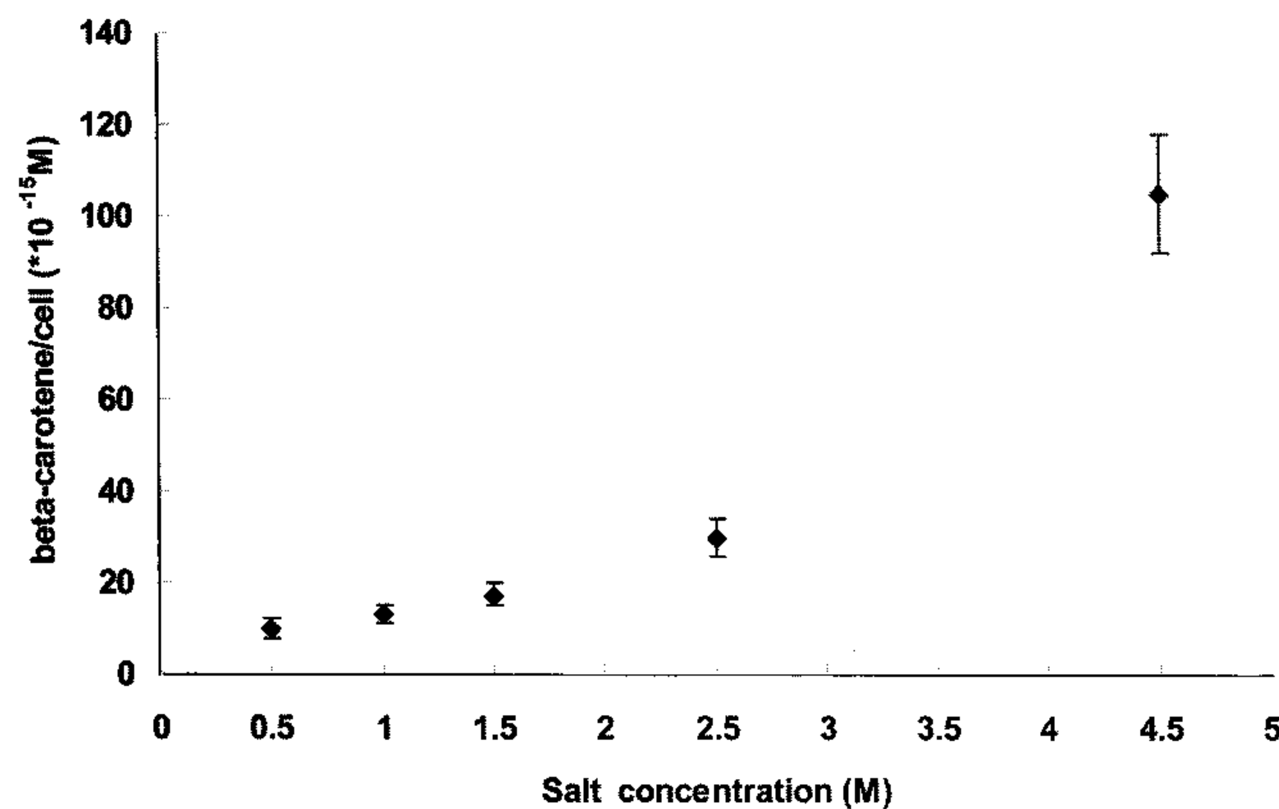

Fig. 4. Accumulation of cellular β-carotene content in the strain *Dunaliella salina* KCTC10654BP.

South Korea might not belong to the species *D. salina*. Consequently, the pigment content of the cells was investigated by use of HPLC for cells grown under different salinities. Fig. 4 compares the amounts of β-carotene per cell for cells of the new *Dunaliella* strain cultivated at various salinities. The amount of β-carotene per cell was relatively low in cells grown at salinities between 0.5 to 2.5 M NaCl. However, when cells were cultivated at 4.5 M NaCl, they contained about a ten-fold higher cellular level of β-carotene as compared with cells grown at optimal salinity. This result demonstrated that cells of the new Korean strain of *Dunaliella* did overaccumulate β-carotene in response to salt stress, thereby fulfilling the biochemical characteristic of the species *D. salina*. However, the unique feature of the new Korean strain is that, for an as of yet unknown reason, its cellular levels of secondary β-carotene were much lower than in other *D. salina* strains.

Molecular Analysis

The internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA are in widespread use for molecular phylogeny studies and it was shown previously that the ITS2 region is specific for species [1, 9, 14]. Furthermore, in addition to the morphological characteristics and the biochemical marker of carotenoid overaccumulation, more recently, nuclear rDNA ITS1 and ITS2 sequences have been used for phylogenetic analysis of the *Dunaliella* species [19, 20]. These studies showed that all carotenogenic *D. salina* strains fell into one major clade and that some noncarotenogenic strains, such as *D. salina* UTEX200, were suggested to not be members of the species *D. salina*. Because the ITS sequences are useful in delineating strains at the species level, in this study, the ITS region was chosen as a molecular marker over the 18s rDNA sequence.

In order to obtain additional sequences for a more detailed comparative study of the new Korean strain with other strains of the species *D. salina*, this study sequenced the ITS1 and ITS2 regions of three more *D. salina* isolates obtained from different culture collections or isolated from their natural habitat (Table 1) and the ITS2 region of the strains *D. salina* DCCBC27 and *D. salina* SAG184.80 (Table 2).

Table 2 compares the number of nucleotides within the ITS1 and ITS2 regions for the various *D. salina* isolates. For all *D. salina* isolates, the ITS1 region contains between 209 and 212 nucleotides. Although the ITS2 sequences for *D. salina* CCAP19/18, *D. salina* (China), *D. salina* (Mexico), and *D. salina* (CONC001) deposited into the NCBI database were incomplete, a comparison with other ITS2 sequences of various *Dunaliella* species showed that only the first four conserved nucleotides (AATC) were missing. For further analysis, adding of the conserved nucleotides AATC

Table 2. Comparison of the number of nucleotides found in the ITS1 and ITS2 regions of the various *D. salina/bardawil* strains and their corresponding GenBank accession numbers.

Species/Isolate	ITS1 (bp)	Accession No.	ITS2 (bp)	Accession No.
D. salina CCAP19/18	209	AF546097	230	AF546098
D. salina China	209	AF546095	230	AF546096
D. salina Mexico	210	AF546093	229	AF546094
D. salina CONC-001	209	AF546091	236	AF546092
D. salina CONC-006	210	AF313424	229	AF313425
D. salina CONC-007	210	AF313426	235	AF313427
D. salina UTEX LB1644	210	AF313428	236	AF313429
D. salina SAG 184.80	-	-	227	AY577766
D. salina AC144 (=CHL10)	211	AY545542	228	AY49441
D. salina DCCBC1	210	AY545543	230	AY549442
D. salina DCCBC27	-	-		
D. salina KCTC10654BP	210	AY512973	230	AY512973
D. bardawil UTEX LB2538=ATCC 30861	209	AF313430	231	AF313431
D. salina UTEX200	209	AF313422	227	AF313423
D. salina sp. 006 Coleman & Brown	-	-	227	AF033278
D. (salina) sp.	212	AJ496573	-	-

could artificially complete these sequences. In contrast to ITS1, comparison of the length of the ITS2 region revealed heterogeneity in the length of the sequences: Two different groups of ITS2 were identified within the *D. salina* isolates that were either 227–231 nucleotides or 235–236 nucleotides long. This heterogeneity in the length of the two groups of ITS2 may suggest the existence of two different subspecies of *D. salina*.

Fig. 5 shows a phylogenetic tree based on the analysis of the combined ITS1 and ITS2 sequences. Strains of *D. salina* fall into different clades, confirming previous results [19, 20] that demonstrated that some strains were previously misidentified. In addition, the major clade of known carotenogenic *D. salina* strains (labeled as 2A and 2B in Fig. 5) includes the new strain of *Dunaliella* from Korea, confirming that this new isolate belongs to the species *D. salina*.

Overall, the results of this study demonstrated that morphological and biochemical characteristics alone might be misleading when used for classification of strains belonging to the species *D. salina* (Teod). The combined morphological, biochemical, and phylogenetic analyses presented in this study demonstrated that even phylogenetically closely related strains might have significant morphological differences. Therefore, use of only morphology for identification would lead to erroneous classification of new *D. salina* isolates. At present, only a joint morphological, biochemical, and molecular analysis appears to be a reliable basis for classification of species within the genus *Dunaliella*. Such unambiguous classification of algal species is essential for any biodiversity studies. In addition, the new *Dunaliella salina* isolate from Korea presents an opportunity for further comparative study of the molecular mechanism(s) that determine the level of carotene accumulation in the species *D. salina*.

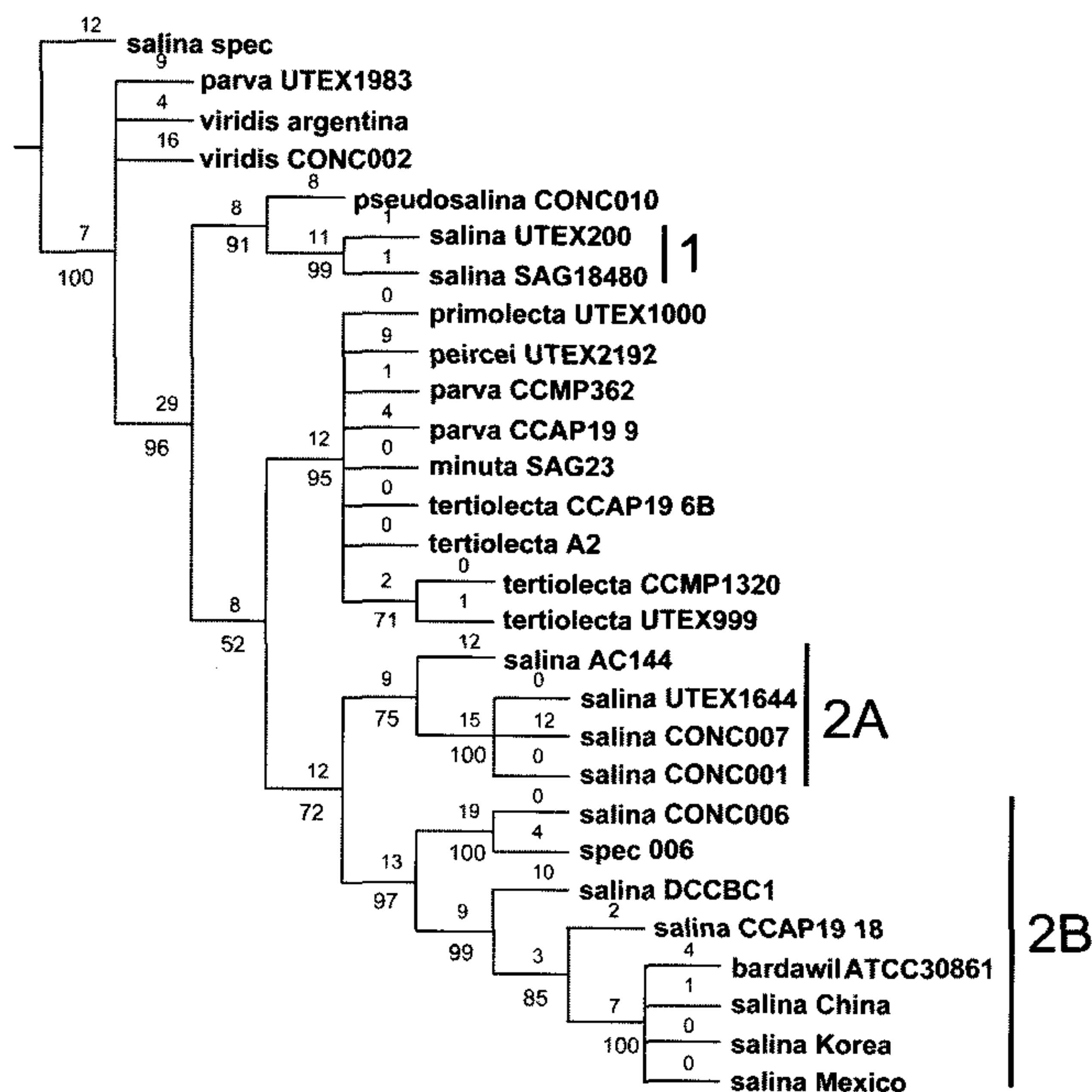

Fig. 5. Phylogram based on maximum parsimony analysis of various available ITS1 and ITS2 sequences from halotolerant *Dunaliella* isolates.

The sequences were either obtained from the NCBI database or through amplification using PCR in this laboratory with subsequent sequencing. The tree shown is the strict consensus of 34 maximum parsimony trees at 267 steps. The strain *D. (salina)* sp. was considered as the outgroup, since the sequence analysis showed that its classification was erroneous and that it might not belong to the genus *Dunaliella*. Tree length, 267 steps; consistency index (CI)=0.68; retention index (RI)=0.85. Numbers above branches are the assigned length, using fast optimization on the strict consensus tree in WinClada. Numbers below branches are jackknife values from a NONA analysis. Strains of *D. salina* fall into two different clades labeled as 1 and 2. Strains included in clade 1 are mistakenly named as *D. salina*. Only clade 2 includes strains that are carotenogenic and thus fulfill the species description for *D. salina*. In addition, note that clade 2 is divided into the subclades 2A and 2B, indicating the existence of two subspecies.

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