Full Length Research Paper

Isolation and molecular identification of β-carotene producing strains of *Dunaliella salina* and *Dunaliella bardawil* from salt soil samples by using species-specific primers and internal transcribed spacer (ITS) primers

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Dunaliella salina and *Dunaliella bardawil* are unique species of the genus *Dunaliella* that produce large amounts of β-carotene when cultivated under appropriate conditions. These include high light intensity, high sodium chloride concentration, nitrate deficiency and extreme temperatures. Under these conditions, only *D. salina* and *D. bardawil* can accumulate β-carotene to as much as 10% of the cellular dry weight. Because the morphological characterization is based on the environmental factors, the *Dunaliella* will change the shape, so identification and differentiation of *Dunaliella* species by morphology is very difficult. In this research study, we isolated, identified and discriminated the different *Dunaliella* β-carotene producing strains from salt soil samples, by using 18S rDNA and internal transcribed spacer (ITS) gene sequences. The soil samples were collected from four different provinces of the North Eastern part of Thailand−namely: UdonThani, BuriRam, AmnartCharoen and Chaiyaphum. Among the four isolates, only BuriRam KU01 and UdonThani KU01 were *D. salina* and *D. bardawil*, respectively whereas AmnartCharoen KU01 and Chaiyaphum KU01 were not these *Dunaliella* species. At 4 M NaCl, with deficiency of nitrate (KNO_3) and phosphate (KH_2PO_4) in the medium, the *D. salina* strain BuriRam KU01 produced β-carotene at the level of 56.25 ± 0.97 pg·cell^{-1} and *D. bardawil* strain UdonThani KU01 produced β-carotene at the level of 52.91 ± 0.29 pg·cell^{-1} at the 25th day after inoculation. The 18S rDNA and ITS sequences of *D. salina* strain BuriRam KU01 and *D. bardawil* strain UdonThani KU01 were submitted to the National Center for Biotechnology Information (NCBI) database with accession numbers of JN052202, JN052203, JN034031 and JN052204, respectively. By using the species-specific primers and ITS primers the β-carotene producing strains of *Dunaliella* was identified.

Key words: 18S rDNA, β-carotene, carotenoid, *Dunaliella bardawil*, *Dunaliella salina*, internal transcribed spacer (ITS), salinity.

INTRODUCTION

The green unicellular flagellate *Dunaliella salina* algae have been classified by Teodoresco (1905). A unique character of *Dunaliella* is withstanding in high salinities, that is, 0.1 to 6.0 M NaCl (Ginzburg and Ginzburg, 1981). There are many species to be identified by taxonomic studies. Some strains of *Dunaliella* can produce high

amount of β-carotene with economic value (Ben-Amotz and Avorn, 1982, 1983, 1988). *Dunaliella* carotene is widely used as a yellow colorant for food products such as confectionery, beverages, noodles and health foods. It is also employed for nutritional reinforcement, as a vitamin A precursor. β-Carotene has been applied as anticancer and antioxidant properties (Hemaiswarya and Double, 2006; Raja et al., 2007a). Although the isolation and identification of *Dunaliella* by conventional methods have been carried out (Massyuk, 1965), neither physio-logical nor morphological techniques provide clarification among the species. Therefore, even today, identification is difficult and time consuming among halophilic and carotenogenic *Dunaliella* species in both green and red algal stages. In salt saturated lagoons, among the other species of *Dunaliella*, *D. salina* and *D. salina/ bardawil* are identified as dominant species of the genus to enrich β-carotene when subjected to extreme conditions, such as high light intensity, nutrient deprivation, high salinity, and extreme temperatures (Ben-Amotz et al., 1982; Ben-Amotz and Avron, 1983; Borowitzka et al., 1990; Shaish et al., 1993; Ben-Amotz, 1996; Krol et al., 1997; Kleinegris et al., 2009). However, there is confusion in distinguishing *D. salina* and *D. bardawil* using morpho-logical characteristics because their characters depend on environmental factors (Borowitzka and Borowitzka, 1988; Borowitzka and Siva, 2007).

Molecular identification provides a useful tool to distin-guish between inter and intra-specific morphologically similar species (Olmos et al., 2000; Gomez and Gon-zalez, 2004) and mixed populations (Hernandez and Olmos, 2006; Garcia and Olmos, 2007). Intra-species identification from community members without requiring their cultivation, can avoid some selective biases asso-ciated with pure culture methods (DeLong et al., 1989). A gene specific primer is a tool to identify species from culture collections or from natural environments (Ki and Han, 2005).
Olsen et al. (1986) identified and classified organisms based on conserved and variable regions of 18S rDNA which is commonly used for taxonomic studies. Also, 18S rDNA has been used for fingerprinting profiles as an indicator for β-carotene hyper-producing species (Olmos et al., 2000; Olmos-Soto et al., 2002; Raja et al., 2007b; Olmos et al., 2009). Ribosomal spacer sequences, inclu-ding ITS regions, have also been frequently utilized for discrimination of genetic relatedness and to study phylo-geny and taxonomy of *Dunaliella* (Coleman et al., 1994; Coleman and Mai, 1997; Gonzalez et al., 1999, 2001; Gomez and Gonzalez, 2001, 2004). Based on the arran-gement of the intron, they had identified a novel18S rDNA gene arrangement along with distinct ITS regions among *Dunaliella* species (Hejazi et al., 2010).

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In this research study, by using 18S rDNA and ITS, we demonstrated that each β-carotene producing species has a different 18S rDNA and ITS fingerprint profiles. From these two fingerprint profiles, the β and non-β-carotene producing species of *Dunaliella* can be clearly and easily discriminate even at the green stage.

MATERIALS AND METHODS

Isolation of algae from soil sample

Modified *Dunaliella* hypersaline medium from the original medium recipe from Weldy and Huesemann (2007). Micronutrients concentration was modified as follows, 150 mM H_3BO_3, 10 mM $MnCl_2$, 0.8 mM $ZnCl_2$, 0.3 mM $CuCl_2$, 2 mM Na_2MoO_4, 2 mM $NaVO_3$ (heat to dissolve) and 0.2 mM $CoCl_2$. After autoclaving, the medium was allowed to cool down to room temperature and then 0.5 M $NaHCO_3$ was filtered, sterilized and added to the medium.

Two grams of the collected soil samples were taken in a test tube and then Modified *Dunaliella* hypersaline medium, with 0.86 M NaCl was added to the tube and mixed thoroughly. After that the tubes were kept under the white fluorescent light of 52.84 μmol $m^{-2} \cdot s^{-1}$ photosynthetic photon flux (PPF) density and were daily shaken for 1 month. One milliliter of the grown algae was transferred to the *Dunaliella* hypersaline medium with 0.86 M NaCl containing 0.02 $g \cdot L^{-1}$ chloramphenicol, and 200 $mg \cdot L^{-1}$ penicillin G for 20 days. The grown algae was streaked on Petri plates containing medium with the same antibiotics. The plates were incubated under 52.84 μmol $m^{-2} \cdot s^{-1}$ PPF provided by the white fluorescent lamps, and then the algae were allowed to grow for the single colony isolation. The single colonies were isolated from the plate and then transferred to the 1.5 M NaCl *Dunaliella* hypersaline medium. Four pure cultures of the isolated *Dunaliella*− namely: AmnartCharoen KU01, BuriRam KU01, Chaiyaphum KU01 and UdonThani KU01 were used as initial strains.

These above culture soil samples were collected from the North Eastern part of Thailand which covers a total area of approximately 170,000 km^2 and lies between 14° 14' to 18° 27' North latitude and 101° 0' and 105° 35' East longitude (Figure 1).

Culture condition

The pure culture obtained was grown in 250 mL of modified *Dunaliella* hypersaline medium. Then, the culture was incubated at 25 ± 2°C ambient temperature with 14 $h \cdot d^{-1}$ photoperiod at 52.84 μmol $m^{-2}s^{-1}$ PPF provided by white fluorescent lamps, for 15 days.

Genomic DNA extraction

Genomic DNA extraction of *Dunaliella* was carried out by slight modification of the method used for *Escherichia coli* (Sambrook et al., 1989). The protocol is as follows: 250 mL of 20-day-old green algae culture (cell density approximately 7 × 10^3 cells·mL^{-1}) was centrifuged for 15 min at 5000 rpm, and the supernatant was discarded. The microalgal cells were resuspended in 20 mL of Buffer S (100 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 1.5 M NaCl and 1% CTAB), and 100 μL proteinase K (10 mg mL^{-1}) were immediately added. The mixture was then thoroughly vortexed. 2 mL of 20% sodium dodecyl sulphate (SDS) were added, mixed gently by inverting, and then incubated at 65°C for 1 h with mixing, by inverting every 15 min. An equal volume of chloroform was added and mixed thoroughly by inverting for 5 min, then spun at 5000 rpm for 20 min. The supernatant was transferred to a new tube and 0.6 volume of isopropanol was added. It was mixed gently
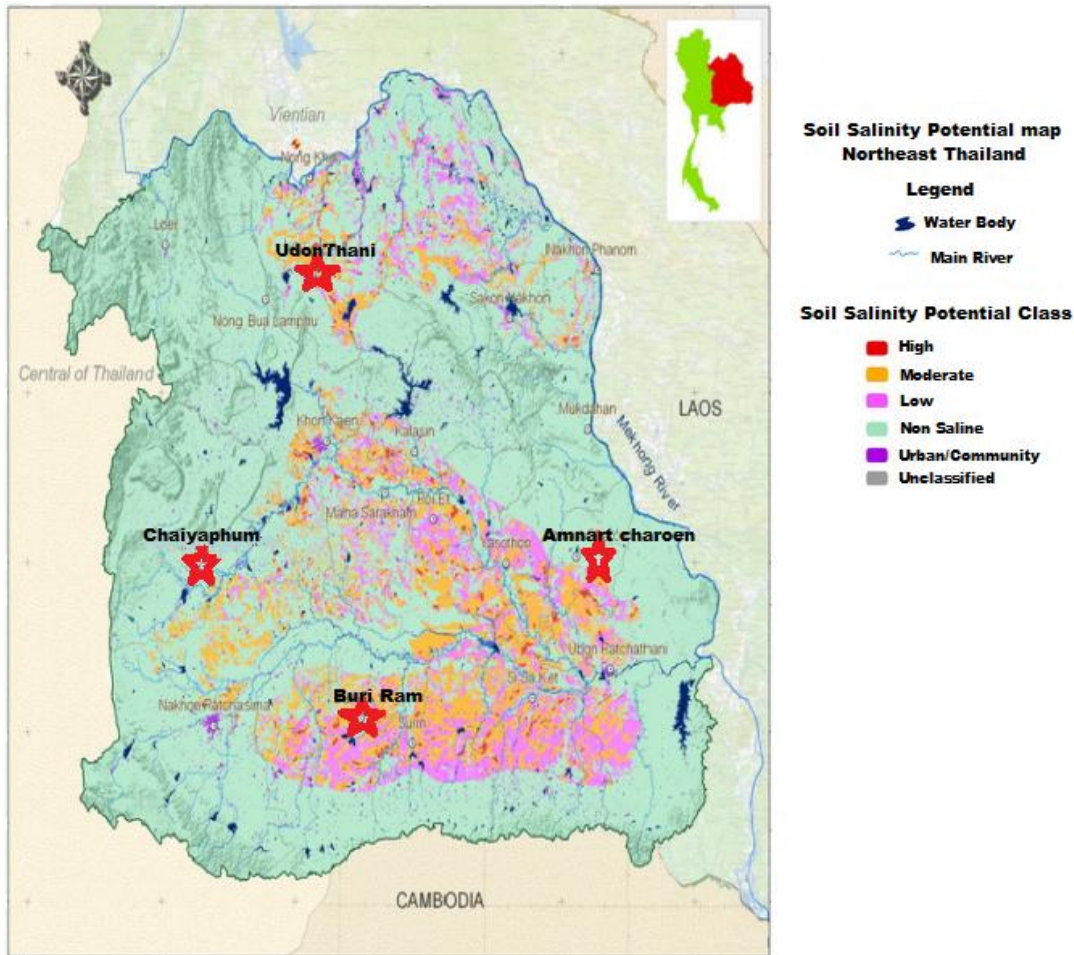
Figure 1. Map showing soil salinity in the North Eastern part of Thailand. Four salt soil samples were collected from BuriRam, UdonThani, Chaiyaphum and AmnartCharoen provinces.

by inverting. Then the tube was kept overnight at 4°C and then centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the DNA pellet was washed with 70% ethanol, air dried and finally dissolved in nuclease-free water and stored at -20°C.

Primers for the amplifications of the 18S rDNA gene and the ITS region

MA1 (5'-CGGGATCCGTAGTCATATGCTTGTCTC-3') and MA2 (5-GGAATTCCTTCTGCAGGTTCACC-3') primers were designed from the exons region and were used to amplify the conserved region of 18S rDNA. The species-specific primers DSs (*D. salina*) (5'-GCAGGAGAGCTAATAGGA-3'), DBs (*D. salina/bardawil*) (5'-GGGAGTCTTTTTCCACCT-3') and DPs (*D. parva*) (5'-GTAGAGGGTAGGAGAAGT-3') were designed from intron I of 18S rDNA, where the most variable sequence is contained (Olmos et al., 2000; Olmos et al., 2002). The two primers AB1 (5'-AATCTATCAATAACCACACCG-3') and AB2 (5'-TTTCATTCGCCATTACTAAGG-3') were designed from conserved sequences flanking the ITS region in *Dunaliella* species. These primers cover nucleotides 1 to 21 of ITS1 and 80 to 100 of the 28S rDNA gene, respectively (Hejazi et al., 2010).

Polymerase Chain Reaction (PCR) conditions for the amplification of the 18S rDNA gene and the ITS region

PCR was carried out in 20 µL reactions containing 50 ng genomic DNA, 1× Taq dream buffer (Fermentas), 200 µmol dNTPs mix (Fermentas), 1.0 Units Taq DNA polymerase (Invitrogen) and 200 ng MA1 and MA2 conserved primers. To To identify the species, the PCR was performed using species-specific primers and MA2 conserved primers (DSs-MA2, DBs-MA2 and DPs-MA2) and the reactions were carried out in the same reaction volume. All PCR amplifications were executed by using a Px2 Thermo cycler (Thermo Electron Corporation). The PCR conditions for different primers are listed in (Table 1)

Gel electrophoresis and photography

The amplified PCR products were fractionated by gel electrophoresis in 1.5% (w/v) agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0) and the gels were stained with 0.5 mg mL^{-1} ethidium bromide. The banding patterns were documented using BioRad (Bio Rad, UK). The molecular weights of the amplified fragments were calculated by comparing with molecular weights of DNA markers (1 kb DNA ladder).

Table 1. PCR conditions for the amplification of 18S rDNA and ITS by using different primers.

PCR step	MA1 and MA2	DSs and MA2	DBs and MA2	DPs and MA2	AB1 and AB2
Initial denaturation	95°C for 5 min	94°C for 3 min	94°C for 3 min	94°C for 3 min	95°C for 5 min
Denaturation	95°C for 1 min	95°C for 30 s	95°C for 30 s	95°C for 30 s	95°C for 1 min
Annealing	56.6°C for 1 min	55.3°C for 30 s	48°C for 30 s	50°C for 30 s	50°C for 50 s
Extension	72°C for 2 min	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1 min
Final extension	72°C for 10 min	72°C for 7 min	72°C for 7 min	72°C for 7 min	72°C for 10 min
Number of cycles	30	35	35	35	30

Table 2. List of *Dunaliella* strains investigated in this study. The 18S rDNA & ITS region sequences of the *Dunaliella salina* BuriRam KU 01 and *Dunaliella bardawil* UdonThani KU 01 isolates were submitted to NCBI database and recorded with accession numbers of JN052202 and JN052203 & JN034031 and JN052204, respectively.

Dunaliella strain	Accession No. (18S rDNA)	Accession No. (ITS region)
Dunaliella bardawil	AF150905	DQ116744
Dunaliella parva	M62998	DQ116746
Dunaliella salina CCAP 19/3	EF473743	EF473744
Dunaliella salina CCAP 19/18	EF473745	-
Dunaliella salina Ds18S1	-	FJ360756
Dunaliella salina Ds18S3	-	FJ360758
Dunaliella salina BuriRam KU 01	JN052202	JN034031
Dunaliella bardawil UdonThani KU 01	JN052203	JN052204

Elution of the amplified DNA from the agarose gel

The amplified band was cut from the gel and the elution was performed using a DNA extraction kit (Fermentas) according to the manufacture's protocol.

Sequencing and alignment

The MA1-MA2 and AB1-AB2 PCR products from *Dunaliella* species were sequenced. The multiple alignments of the sequence were performed by using the BioEdit program. *Dunaliella* gene bank sequences comparison analysis was carried out with the Basic Local Alignment Search Tool (BLAST) program from National Center for Biotechnology Information (NCBI) by using the 18S rDNA gene sequence with ~2500 and ~2100 bp, and the ITS region with ~700 bp.

Dendrogram construction of sequences

To analyze the dendrogram of the isolated BuriRam KU01 and UdonThani KU01 strains, alignment of the sequences was performed with the sequences of different *Dunaliella* species which were submitted in the NCBI database (Table 2). MEGA5 software was employed to construct a dendrogram, by using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), the evolutionary distances were computed using the Maximum Composite Likelihood model and reliability of the branches was assessed by bootstrapping the data with 1000 replicates.

Salt stress for β-carotene production

The BuriRam KU01 and UdonThani KU01 samples were cultivated in liquid medium containing five different NaCl concentrations (0.5, 1.0, 2.0, 3.0 and 4.0 M) with deficiency of nitrate (KNO_3) and phosphate (KH_2PO_4) in the medium, incubated under 25 ± 2°C ambient temperature with 14 h d^{-1} photoperiod provided by white fluorescent lamps, at 72.84 µmol $m^{-2}s^{-1}$ PPF. The flasks were shaken at 110 rpm in the orbital shaker. All of the experiments began by inoculating 50 mL of the flask volume with algae in the logarithmic growth phase after 15 days (the data was not shown). Then the cells were grown in the modified *Dunaliella* hypersaline growth medium with different NaCl concentrations as mentioned above to obtain the target salinity. Salt stress conditions were evaluated to determine the maximum β-carotene production. Carotenoid extraction was carried out after 25 days of cultivation. β-Carotene production ability of the desired samples was evaluated according to the protocol provided by the method of Celekli and Donmez (2006).

Cell counts

To compare cell growth in different salinities, cell counting was done by using a light microscope and haemocytometer (NEU-BAUER) at the 25th day of cultivation.

Soil analysis

For physical and chemical properties of the soil samples, soil texture, percentage of particle size, organic matter, pH, phosphorus, calcium, magnesium and potassium were analyzed. Electrical conductivity (EC) was used to represent soil salinity and expressed in units of milliSiemens per centimeter (mS cm⁻¹). One gram of soil sample was added to 5 mL of double distilled water and allowed to stir for half an hour and filtered with Whatmann® No.1 filter paper. Then, the EC was measured by using an EC meter.
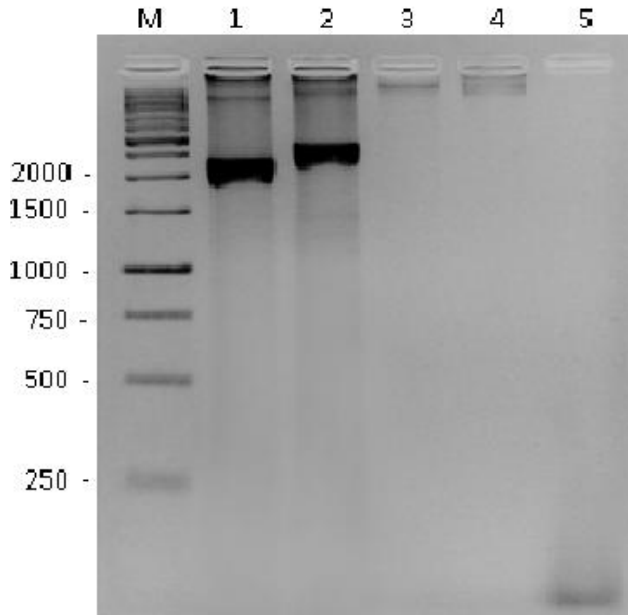
showed 97% homology with *D. bardawil* strain UTEX LB 2538, with the maximum identity of 99%.

The sequence alignment was performed by using the BioEdit sequence alignment editor and the results are shown in Figure 3. The 18S rDNA sequence of the two isolates was submitted to NCBI in the names of *D. salina* BuriRam KU01 and *D. bardawil* UdonThani KU01, and the accession numbers of these isolates are JN052202 and JN052203, respectively.

The dendrogram derived by using 18S rDNA sequence of the BuriRam KU01, UdonThani KU01 and other *Dunaliella* species sequences were grouped into two major clusters−namely: A and B. Cluster A consists of *D. salina* 19/18, *D. salina* 19/3, BuriRam KU01 and UdonThani KU01. Cluster B consists of *D. bardawil* and *D. parva*. The results of the bootstrap analysis of UPGMA tree are displayed in Figure 4.

PCR amplification by using species-specific primers

By using the species-specific primers, two different DNA fragments were found in the amplified product. With DSs-MA2 primers, the BuriRam KU01 DNA sample amplified a ~700 bp DNA fragment and there was no amplification with DBs-MA2 and DPs-MA2 primers (Figure 5A). On the other hand, the UdonThani KU01 DNA sample amplified a ~1000 bp fragment with DBs-MA2 primers and there was no amplification with DSs-MA2 and DPs-MA2 primers (Figure 5B). These clearly showed that the isolated strains from BuriRam KU01 and UdonThani KU01 DNA soil samples belong to the species *D. salina* and *D. bardawil*, respectively.

PCR amplification of ITS region

In addition to the 18S rDNA gene, the Internal Transcribed Sequence (including ITS1, 5.8 rDNA and ITS2) of the isolates, was amplified and sequenced for further confirmation. The PCR amplification of BuriRam KU01 and UdonThani KU01 DNA samples with ITS specific primers (AB1-AB2) resulted in the production of a single band, with a size of ~700 bp (Figure 5C). There is no difference in the size of the band between these two different species. Then, the amplified PCR product was sequenced, and the sequence was aligned with 6 different strains whose ITS sequences were fully recorded at NCBI (Table 2).

Based on blasted sequence in the NCBI, the BuriRam KU01 ITS region sequence showed 97% homology with *D. salina* strain SAG 42.88 and the UdonThani KU01 sequence showed 92% homology with *D. bardawil* strain. The sequence alignment was performed by using the BioEdit sequence alignment editor and the results are shown in Figure 6. The ITS region sequences of the two salt soil sample isolates were submitted and recorded with the accession numbers of JN034031 and JN052204

Figure 2. Amplification of DNA by using 18S rDNA conserved primers (MA1-MA2). M = 1 Kb DNA ladder. Lane 1: ~2100 bp amplification of BuriRam KU01 DNA sample; lane 2: ~2500 bp amplification of UdonThani KU01 DNA sample; lanes 3 and 4: No amplification of Chaiyaphum KU01 and Amnart Charoen KU01 DNA samples and; lane 5: Negative control (without DNA).

Statistical analysis

Each result showed the mean of three replicated studies. Statistical analysis and graphical illustrations were performed by using the SAS version 9 and Microsoft Excel programs, respectively. The statistical significance was determined at 95 or 99% level of confidence limit.

RESULTS

PCR amplification of the 18S rDNA gene

The PCR amplification was performed by using MA1-MA2 primers and confirmed the genus of our isolates. Among the four isolates, the PCR with MA1-MA2 primers allowed the amplification of 18S rDNA and resulted in production of ~2100 and ~2500 bp DNA bands in Buri-Ram KU01 and UdonThani KU01 DNA samples, respectively (Figure 2). This indicated that only BuriRam KU01 and UdonThani KU01 strains belong to the genus *Dunaliella.* But there was no amplification in the Chaiya-phum KU01 and AmnartCharoen KU01 strains. This indicated that these strains did not belong to the genus *Dunaliella.* The amplified PCR product was sequenced and then aligned with four different strains whose 18S rDNA sequences were fully recorded at NCBI (Table 2).

When the obtained sequences were blasted in the NCBI, the BuriRam KU01 sequence showed 98% homology with *D. salina* strain CCAP 19/30, with the maximum identity of 98%, while the UdonThani KU01 sequence

Figure 3. (A) Amplification of BuriRam KU01 DNA by using specific-conserved primers (DSs-MA2, DBs-MA2 and DPs-MA2). Lane M = 1 Kb DNA ladder. Lane1: ~700 bp amplification of BuriRam KU01 DNA sample with DSs-MA2; lanes 2 and 3: No Amplification of BuriRam KU01 DNA sample with DBs-MA2 and DPs-MA2. (B) Amplification of UdonThani KU01 DNA with specific-conserved primers (DSs-MA2, DBs-MA2 and DPs-MA2). Lane M=1 Kb DNA ladder. Lane1: ~1000 bp amplification of UdonThani KU01 DNA sample with DBs-MA2; lanes 2 and 3: No amplification of UdonThani KU01 DNA sample with DSs-MA2 and DPs-MA2; (C) ITS amplification with the primer (AB1-AB2). Lane M = 1 kb DNA ladder. Lanes1 and 2: ~700 bp amplification of BuriRam KU01 and UdonThani KU01 DNA samples.
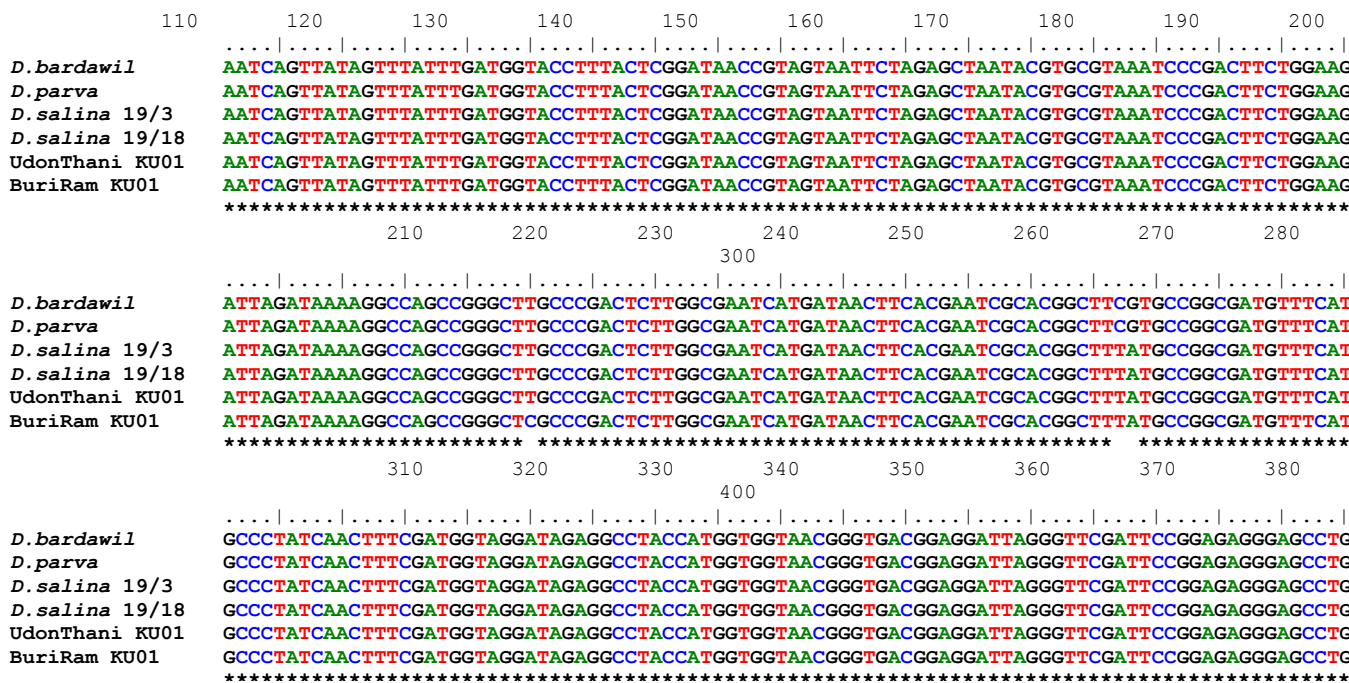
Figure 4. 18S rDNA sequence alignment of *Dunaliella* species: BuriRam KU01, UdonThani KU01 and other *Dunaliella* species. Data for other species were gathered from NCBI. The conserved regions of the gene are shown in asterisk.

Figure 5. Dendrogram showing the clustering among *Dunaliella* species BuriRam KU01 and UdonThani KU01, and species of *Dunaliella*. The tree is based on the 18S rDNA region and was conducted by using UPGMA method. Bootstrap values were calculated from 1000 replicates.

in NCBI database.

The relationship between the isolated species and the other *Dunaliella* species, based on ITS region, is shown in Figure 7. The cluster analysis showed a dendrogram with two clusters; cluster A formed with all *D. salina* and BuriRam KU01 and cluster B was composed of *D. bardawil*, *D. parva* and UdonThani KU01.

Cell count and β-carotene production ability

Two amplified isolates were grown in modified *Dunaliella* hypersaline medium with different concentrations of NaCl, ranging from 0.5 to 4.0 M, with deficiency of nitrate (KNO_3) and phosphate (KH_2PO_4) in the medium. The cell number was high in 0.5 M NaCl medium and was low in 4.0 M NaCl medium in both strains at 25th day after inoculation. For the β-carotene quantification, the molecularly identified *D. salina* strain BuriRam KU01, and *D. bardawil strain* UdonThani KU01 produced high β-carotene of 56.25 ± 0.97 and 52.91 ± 0.29 pg cell^{-1} in 4.0 M NaCl medium after 25 days of inoculation, respectively (Table 3).

Soil analysis

For physical properties, the soil sample from UdonThani composed of more silt and clay when compared to BuriRam, AmnartCharoen and Chaiyaphum. Moreover, the chemical properties of the UdonThani soil sample showed higher organic matter, potassium, magnesium and EC value (Table 4).

DISCUSSION

According to the study of Olmos et al. (2000, 2002, 2009), by using conserved primer MA1-MA2, PCR ampli-

fied the expected size DNA fragment. This confirms the genus of the isolate as *Dunaliella* species. Taking these results together, the 18S rDNA gene of our BuriRam KU01 amplified with MA1-MA2 = ~2100 bp and DSs-MA2 = ~700 bp (Figures 2 and 5A). It did not amplify with DBs-MA2 and DPs-MA2 species-specific primers. Fascinatingly, the same fingerprinting was shown by *D. salina* M84320 isolated from Chile and reported by Wilcox et al. (1992). Similar result was obtained in *D. salina* BCO2 strain isolated in Mexico (Olmos et al., 2000; Paniagua et al., 2009) and a *D. salina* strain found in India (Raja et al., 2007b). Both strains presented the same 18S rDNA fingerprint. A common characteristic from these *D. salina* strains, in addition to their finger-printing profile, was their β-carotene hyper-production capacity. Since the results which were obtained from the *D. salina* strains isolated from different continents sho-wed the same fingerprinting profile and β-carotene levels; we can be assured that the use of 18S rDNA is a reliable, rapid and sensitive method for classifying these hyper producer strains of *D. salina*. The *D. salina* strains with the fingerprinting profile of MA1-MA2 = ~2100 bp and DSs-MA2 = ~700 bp, belong to the same species, are distributed worldwide, and their β-carotene hyper production capacity is well conserved. Thus, the isolate belongs to the species *D. salina* and has the ability to produce β-carotene (Table 3). UdonThani KU01 of our soil sample isolate produced a fragment of ~2500 bp by using MA1-MA2 primers and produced a fragment of ~1000 bp by using DBs-MA2 species-specific primers (Figures 2 and 5B), there is no amplification with DSs-MA2 and DPs-MA2. The same results were obtained from the UTEX (LB2538) and the *Dunaliella* strain isolated from Baja Mexico (Olmos et al., 2009). Microscopic differentiation between *D. salina* and *D. bardawil* species is difficult and time consuming. However, 18S rDNA of *D. bardawil* has

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                        210       220       230       240       250       260       270       280       290
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D.bardawil     TTTGGTGCTTCCCGGG-TAGCATTAACTTGCTGCTTGGGTT-GGGCTCGGCTGCCCA-TTCATCTGGGTAGTCGGGTTGGTCT--TTGCTAA
D.parva        TTTGGTGCTTCCCGGG-TAGCATTAACTTGCTGCTTGGGTT-GGGCTCGGCTGCCCA-TTCATCTGGGTAGTCGGGTTGGTCT--TTGCTAA
D.salina 19/3  TTTGGTGCTTCCCGGG-TAGCATTAACTTGCTGCTTGGGTT-GGGCTTGGCTGCCCA-TTAATTTGGGTAGTTAGGCTGGTCT--TTACCAA
D.salina 19/18 TTTGGTGCTTCCCGGG-TAGCATTAAGTTGCTGCTTGGGTT-GGGTCCGGCTATCCA-TCAACTTGGGTAGTCGGGCTGGTCC--TTACTAA
D.salina Ds18S3 TTTGGTGCTTCCCGGG-TAGCATTAACTTGCTGCTTGGGTT-GGGCTCGGCTGCCCA-TTCATTTGGGTGGTCGGGTTGGTCT--TTGCTAA
D.salina Ds18S1 TTTGGTGCTTCCCGGG-TAGCATTAACTTGCTGCTTGGGTTTGGGCTCGGCTGCCCA-TTCATTTGGGTGGTCGGGGTGGTCT--TTTCTAA
BuriRam KU01   TTTGGCGCTTCCTGGG-CAGCATTAACTTGTTGCTCGGGTT-GGGCTCGGCTGCCCAATCTCTTTGGGTAGTCGGGTCGGTCTTATTACTAA
UdonThani KU01 TTTGGCGCTTCCTGGGGCAGCATTAACTTGTTGCTCGGGTT-GGGCTCGGCTGCCCAATCTCTTTGGGTAGTCGGGTCGGTCTTATTACTAA
               *****  ****** ***  ******** *** **** ****** ***    **** *** *   ***** ** **  ****   ** ****
                        310       320       330       340       350       360       370       380       390
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
D.bardawil     ACCAAATCAAAACTAAAGCCAAAGTATGTGCTCGGCCTAGCCGTCACATCCTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
D.parva        ACCAAATCAAAACTAAAGCCAAAGTATGTGCTCGGCCTAGCCGTCACATCCTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
D.salina 19/3  ACCAAATCAAAACCTAAAGCCAAAGATATGTGCTCGGCCTAGCCGTCACATCCTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
D.salina 19/18 ACCAAATCAAACCTAAAGCCAAAGATATGTGTTCGGCCTAGCCGTCACATCCTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
D.salina Ds18S3 ACCAAATCAAAACTAAAGCCAAAGTATGTGCTCGGCCTAGCCGTCACATCCTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
D.salina Ds18S1 ACCAAATCAAAACTAAAGGCAAAGTATGTGCTCGGGCTAGCCGTCACATACCAACTGAGACA-CTCTCAACA-CGTATACTT--GCTCTCG
BuriRam KU01   ACCAAACAAAACCTAAAGCCAAAGTATGTGATCGGCCTAGCCGTCACATCCAAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
UdonThani KU01 ACCAAACAAAACCTAAAGCCAAAGTATGTGATCGGCCTAGCCGTCACATCCTAACTGAGACAACTCTCAACAACGGATATCTTGGCTCTCG
               ******  *** ****************** ****************** * ********* ********* ** ***   *  *******
                        410       420       430       440       450       460       470       480       490
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
D.bardawil     AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGCCCAAGGCTTCGGCT
D.parva        AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGCCCAAGGCTTCGGCT
D.salina 19/3  AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGCCCAAGGCTTCGGCT
D.salina 19/18 AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGCCCAATGCTTCGGCT
D.salina Ds18S3 AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGCCCAAGGCTTCGGCT
D.salina Ds18S1 AG--ACGCAGCGAA-TGCG-TACGTAGTGTGA-TTGCAGA--TTCGTGA-TCATCAA-TCTTTGA-CGTAA-TTGCGC---AAGCATCG-CT
BuriRam KU01   AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGTCCGAGGCTTCGGCC
UdonThani KU01 AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAATCTTTGAACGCAAATTGCGTCCGAGGCTTCGGCC
               *  ********** **** ************* *******   * ***** ******* ******* ** ** *****   *  ** *** *
                        510       520       530       540       550       560       570       580       590
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
D.bardawil     GTTTGCCTCAGCGTCGGGTTAATCTCACTCCCCTCCTCCCATAGTGGA--AGCGGGTAAGTGGACCTGGCTGTTC--CAGAGCTTGAGTGCT
D.parva        GTTTGCCTCAGCGTCGGGTTAATCTCACTCCCCTCCTCCCATAGTGGA--AGCGGGTAAGTGGACCTGGCTGTTC--CAGAGCTTGAGTGCT
D.salina 19/3  GTTTGCCTCAGCGTCGGGTTAATCTCACTCCCCATCTCCCACAGCGGAGCAAAGGGTA-GTGGACCTGGCTGTTC--CAGAGGTCGAGTGCT
D.salina 19/18 GTTTGCCTCAGCGTCGGGTTAATCTCACTTCCCCCTTCCCAAAGCGGAGTGTTGGGAAAGTGGACCTGGCTGTTC--CAGAGCTTGAATGCT
D.salina Ds18S3 GTTTGCCTCAGCGTCGGGTTAATCTCACTCCCCTCCTCCCATAGTGGA--AGCGGGTAAGTGGACCTGGCTGTTCCCCAGAGCTTTAGTGCT
D.salina Ds18S1 GTT-GCTCACGCGT-------ATCTCACTCCCTCCT--CCATAGTG-A--AGCGG-TAAGTGGACCTGGCTGT----CAGAGCTT-AGTGCT
BuriRam KU01   GTTTGCCTCAGCGTCGGGTTAATCTCACTAAACCTCTCCTTATG-GGAGCAA-GGCTAAGTGGACCTGGCTGTTC--CAGAGCTTG-GTGCT
UdonThani KU01 GTTTGCCTCAGCGTCGGGTTAATCTCATTAAACCTCTCCTTATG-GGAGCAA-GGCTAAGTGGACCTGGCTGTTC--CAGAGCTTG-GTGCT
               *** **  ****      ****** *   *   * *   * *  ** * *************    *******  ****
                        610       620       630       640       650       660       670       680       690
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
D.bardawil     CTTGGCAATAGCTCTGGATCAGCTGAAGAGTAGAGGCTAGCTCAAGGACCCGTCAAGGGCCGCAACTGGGTAGGCAGCTCACGCTTGCTATT
D.parva        CTTGGCAATAGCTCTGGATCAGCTGAAGAGTAGAGGCTAGCTCAAGGACCCGTCAAGGGCCGCAACTGGGTAGGCAGCTCACGCTTGCTATT
D.salina 19/3  CTCATCAATAGCTCTGGATCAGCTGAAGAGCAGAGGCTAGCTCAAGGACCCGTCAAGGGCCGCAACTGGGTAGGCAGCTCACGCTTGCTATT
D.salina 19/18 CTCGTGAAGGGCTCTGGATCAGCTGAAGAGCAGAGGCTAGCTCAAGGACCCGTCAAGGGCCGCAACTGGGTAGGCAGCTCACGCTTGCTATT
D.salina Ds18S3 CTTAGCAAAAGCTCTGGATCAGCTGAAGAGTAGAGGCTAGCTCAAGGGCCCGTCAAGGGCCGCAACTGGGTAGGCAGCTCACGCTTGCTATT
D.salina Ds18S1 CTTAGCAAAAGCTCTGGATCAGCTGAAGAGTAGAGGCTAGCTCAAGG-CCCGTCAAGG-CCGCAACTGG-TAGGCAGCTCACGCTTGCTATT
BuriRam KU01   CCT-CCAAAGGCTTTGGATCAGCTGAAGAGTAGAGGCTAGCTCAAGGACCCGTCAAGGGCCGCAACTGGGTAGGCAGCAT-TGCTTGCTATT
UdonThani KU01 CCT-TCAAAGGCTTTGGATCAGCTGAAGAGCAGAGGCTAGCTCAAGGACCCGTCAAGGGCCGCAACTGGGTAGGCAGCTT-TGCTTGCTATT
               *     **  *** *************** *************** ********** ********** ********   **********
                        710       720       730       740       750       760       770       780       790
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
D.bardawil     GTTGGCTTGGGACCATGAGCTCGGCCCTCAA-ACAGGACCTCTTTTTTTCTCGACCTGAGCTCAA-GCAAGACCACCCGCTGAACTTAAG
D.parva        GTTGGCTTGGGACCATGAGCTCGGCCCTCAA-ACAGGAACTCTTTTTTTTCTCGACCTGAGCTCAA-GCAAGACCACCCGCCGAACTTAAG
D.salina 19/3  GTTGGCTTGGGACCATGAGCTCGGCCCTCAA-ACAGGAACC-CTCTTTTTTCTCGACCTGAGCTCAA-GCAAGACCACCCGCTGAACTTAAG
D.salina 19/18 GTTGGCTTGGGACCATGAGCTCGGCCCTCAA-ACAGGAACCTCTTTTTTTTCTCGACCTGAGCTCAA-GCAAGACCACCCGCTGAACTTAAG
D.salina Ds18S3 GTTGGCTTGGGACCATGAGCTCGGCCCTCAA-ACAGGAACCTCTTTTTTT-CTCGACCTGAGCTCAA-GCAAGACCACCCGCTGAACTTAAG
D.salina Ds18S1 GTTGGCTTGGGACCATGAGCTCGGCCCTCAAGACAGGAACCTCTTTTTTT-CTCGACCTGAGCTCAAAAGAAAGACCACCCGCTGAACTTAAG
BuriRam KU01   GTTGGCTTGGGACCATGGGCTTGGCCCTCAA-ACAGGACCCTCTTTTTTTCTCGACCTGAGCTCAA-GCAAGACCACCCGCTGAACTTAAG
UdonThani KU01 GTTGGCTTGGGACCATGGGCTTGGCCCTCAA-ACAGGACCCTCTTTTTTTCTCGACCTGAGCTCAA-GCAAGACCACCCGCTGAACTTAAG
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Figure 6. ITS (ITS, 5.8 rDNA and ITS2) sequence alignment of *Dunaliella* species: BuriRam KU01, UdonThani KU01 and other *Dunaliella* species. Data for other species were gathered from NCBI. The conserved regions of the gene are shown in asterisk.
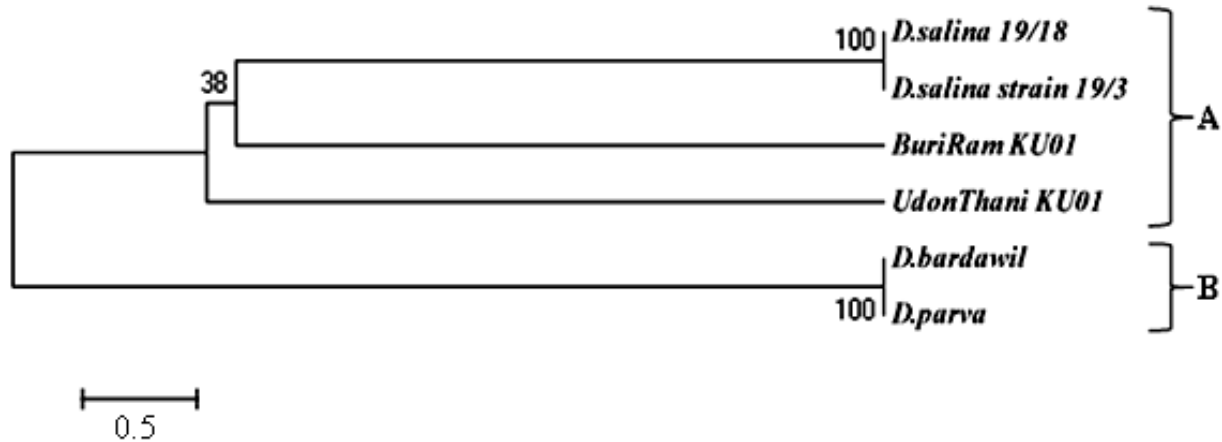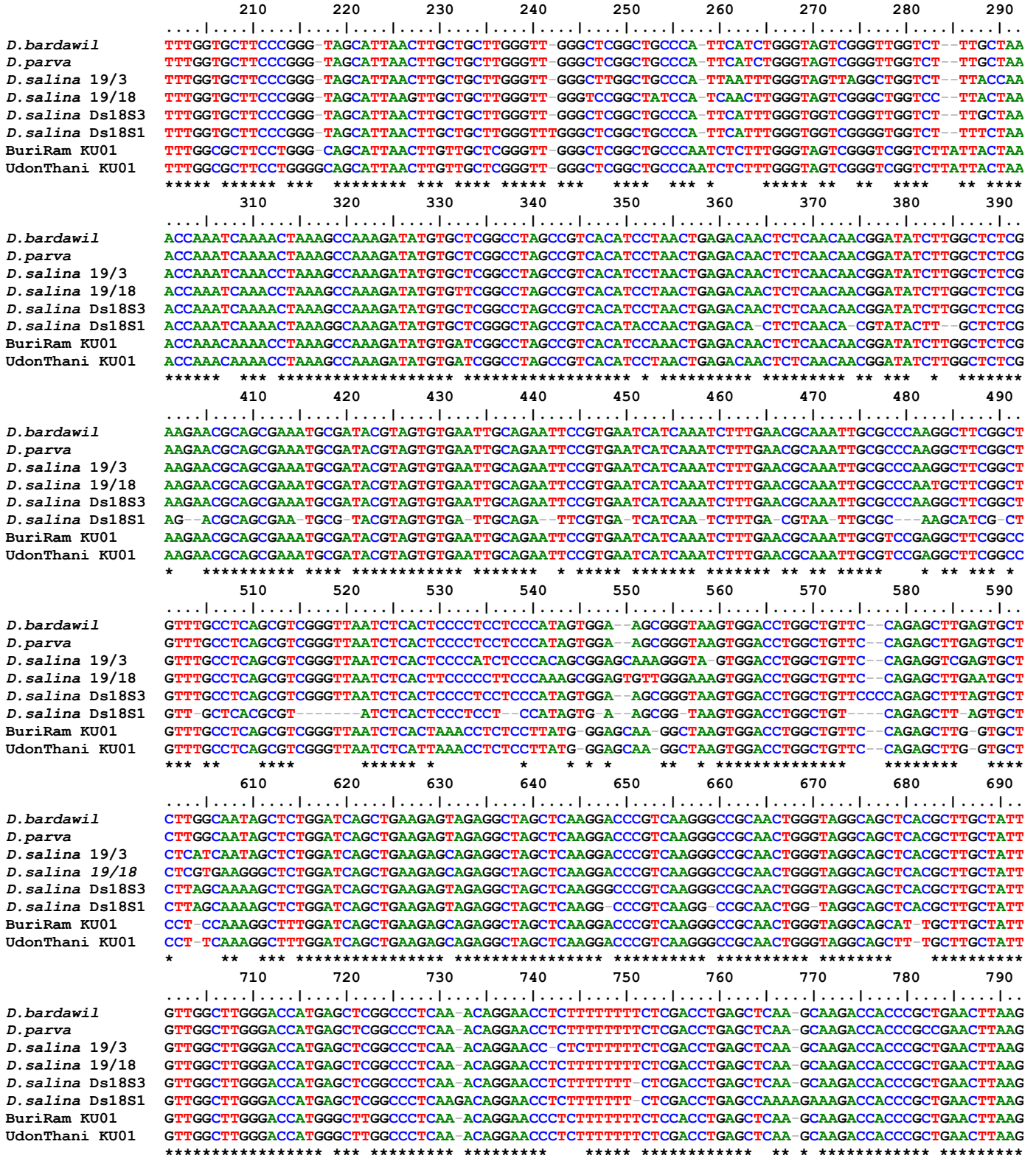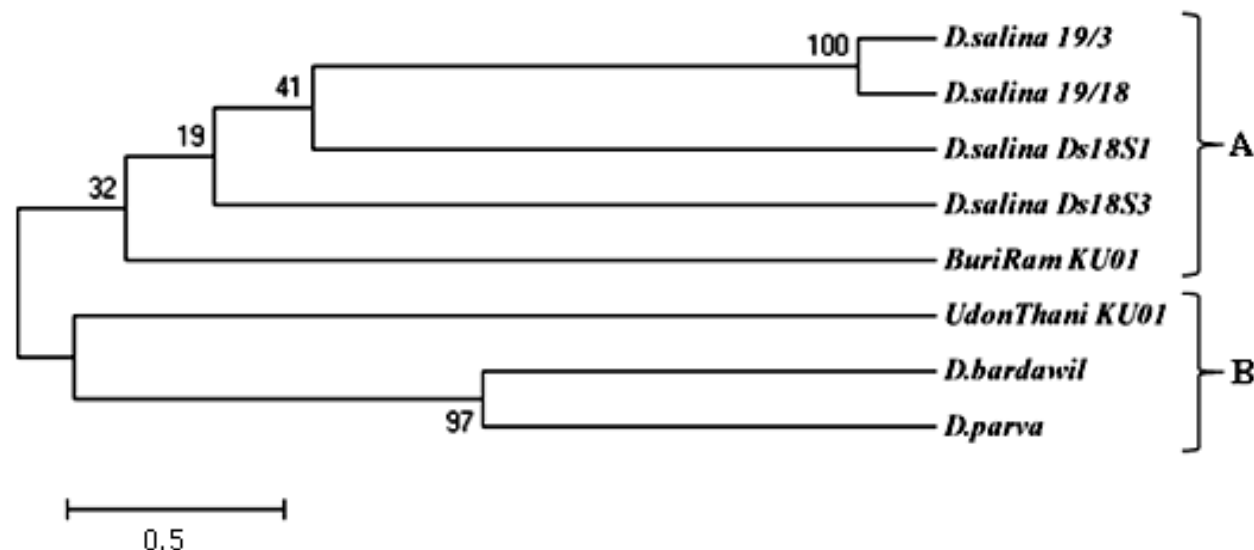
Figure 7. Dendrogram showing the clustering among *Dunaliella* species BuriRam KU01 and UdonThani KU01, and species of *Dunaliella*. The tree is based on the ITS region and was conducted using UPGMA. Bootstrap values were calculated from 1000 replicates.

Table 3. β-Carotene content in algal cells of *D. salina* strain BuriRam KU01 and *D. bardawil* strain UdonThani KU01 cultured in medium containing different salt concentrations. The highest β-carotene production was obtained in 4.0 M NaCl medium in both strains at the 25th day of cultivation.

NaCl (M)	BuriRam KU01				UdonThani KU01			
	Cell number (cell/ml ×10⁶)	Optical density (OD$_{455}$)	β-Carotene (mg/ml)	β-Carotene (ng/cell)	Cell numb. (cell/ml ×10⁶)	Optical density (OD$_{455}$)	β-Carotene (mg/ml)	β-Carotene (ng/cell)
0.5	4.00±0.20^a	0.33±0.01^e	0.026±0.00^e	06.4±0.39^e	3.44±0.06^a	0.25±0.00^e	0.01±0.00^e	02.20±0.04^e
1.0	3.90±0.20^a	0.40±0.01^d	0.043±0.00^d	11.03±0.27^d	2.93±0.06^b	0.31±0.00^d	0.02±0.00^d	07.34±0.22^d
2.0	3.30±0.20^b	0.73±0.02^c	0.119±0.01^c	36.05±0.61^c	2.53±0.06^c	0.46±0.00^c	0.06±0.00^c	22.21±0.46^c
3.0	2.93±0.15^c	0.88±0.03^a	0.153±0.01^a	52.33±0.19^b	2.17±0.12^d	0.67±0.02^a	0.11±0.01^a	49.28±0.94^b
4.0	2.23±0.12^d	0.79±0.02^b	0.133±0.00^b	56.25±0.97^a	1.80±0.10^e	0.63±0.02^b	0.10±0.00^b	52.91±0.29^a
F-test	**	**	**	**	**	**	**	**
C.V. (%)	5.41	3.41	5.24	1.73	3.17	3.05	5.78	1.85

**The treatment means are highly significantly different at $p \leq 0.01$. Means with the different letters in the same column are significantly different at $P \leq 0.05$ by Duncan's new multiple range test (DMTR).

Table 4. Physical and chemical properties of soil samples from UdonThani, BuriRam, Amnartcharoen and Chaiyaphum. The soil sample from UdonThani contains moderate organic matter and phosphorus and high potassium, calcium, magnesium and EC value when compared to other soil samples.

| Soil sample | Soil pH | Particle (%) | | | Texture | Organic matter (%) | Available phosphorus (mg·kg^{-1}) | Available potassium (mg·kg^{-1}) | Available calcium (mg·kg^{-1}) | Available magnesium (mg·kg^{-1}) | EC value (mS·cm^{-1}) |
		Sand	Sandy loam	Clay							
UdonThani	6.1	38	43	19	Loam	2.01	16	900	840	600	14.75±0.17
BuriRam	7.2	68	29	03	Sandy loam	0.36	25	50	1,360	200	6.89±0.03
Amnartcharoen	7.2	78	21	01	Loamy sand	0.35	09	50	720	150	6.22±0.04
Chaiyaphum	6.6	82	15	03	Loamy sand	0.31	18	20	1,040	320	2.73±0.09

an exclusive fingerprinting profile of MA1-MA2 = ~2500 bp/DBs-MA2 = ~1000 bp which are different from *D. salina*. This clearly indicates that the isolated UdonThani KU01 strain is *D. bardawil*, whereas Chaiyaphum KU01 and Amnat-Charoen KU01 are not *Dunaliella* species; because they did not amplify by using MA1-MA2 conserved primers (Hejazi et al., 2010).

According to the dendrogram result of 18S rDNA sequence, BuriRam KU01 isolates belongs to *D. salina*. With the species specific primers (DBs-MA2) it is clearly shown that the UdonThani KU01 belongs to *D. bardawil* and not *D. salina*.

Amplification of the ITS region resulted in the production of a single band with a size of ~700 bp in both soil sample isolated strains (Figure 5C). The ITS region in *Dunaliella* does not show the length difference between the species at the intra or inter-specific levels (Olmos et al., 2009). Even though there is no difference in the length of the amplified product, there is a difference in the ITS sequence, which was reported by Gomez and Gonzalez (2004) and Hejazi et al. (2010). This result is helpful for differentiating the *Dunaliella* species. By the above ITS sequence information, the BuriRam KU01 and UdonThani KU01 are *D. salina* and *D. bardawil*, respectively. From the dendrogram obtained using the ITS sequence, it

is clearly indicated that BuriRam KU01 belongs to *D. salina*, and UdonThani KU01 formed a cluster with *D. bardawil* and *D. parva*.

High organic matter, available potassium and available magnesium is helpful for the growth of the algae and led to the increase of the β-carotene production ability (Borowitzka, 1988; Abalde et al., 1991) because organic matter is a reservoir of nutrients that can be released to the soil. According to McLachlan (1960), magnesium and potassium are required for *Dunailella* growth. In the soil analysis, the result showed that there is high level of high organic matter, available potassium and available magnesium in the UdonThani soil sample and the isolated strain from this soil sample produced high β-carotene. This showed that these nutrients are needed for the growth of β-carotene producing strain in the soil (Table 4).

Among the four isolated strains, the BuriRam KU01 and UdonThani KU01strains were isolated from soil where the salinity was high, when compared with AmnartCharoen KU01 and Chaiyaphum KU01 strains. This clearly confirms that the β-carotene producing strain can withstand high salinity condition (Ginzburg and Ginzburg, 1981). From the salinity map, it is clear that Buri-Ram and UdonThani provinces are located in the high / moderate soil salinity area, whereas Amnart-Cha-

roen and Chaiyaphum province are located in the low/non saline area (Figure 1). This corresponds to the EC value of the soil samples (Table 4). According to Mojaat et al. (2008) *D. salina* CCAP 19/18produced β-carotene 70.0 pg cell^{-1}, whereas the isolated *D. salina* BuriRam KU01 and *D. bardawil* UdonThani KU01 produced only 56.25 ± 0.97 and 52.91 ± 0.29 pg cell^{-1}, respectively. This may be due to the different species of the *Dunaliella* isolates which accumulate β-carotene when grown under different environmental conditions.

The 18S rDNA and the ITS region proposed in this work provide useful techniques to discriminate the two most important carotenogenic species of *Dunaliella* and to make a prompt and exact identification of them. This method is helpful at the beginning of the isolation process even in the green stage.

Conclusion

In this study, the identification of β-carotene producing strains from salt soil by using the 18S rDNA gene size and sequence and the ITS region sequence was demonstrated. In this way, the *D. salina* strain BuriRam KU01 and the *D. bardawil* strain UdonThani KU01 can be easily differentiated. These two soil sample isolates produced

β-carotene at the level of 56.25 ± 0.97 pg cell^{-1} (*D. salina* strain BuriRam KU01) and 52.91 ± 0.29 pg cell^{-1} (*D. bardawil* strain UdonThani KU01), after 25 days of inoculation, respectively. The β-carotene production was enriched when *Dunaliella* was subjected to 4.0 M NaCl. Finally, the β-carotene producing strains of *Dunaliella* were exactly identified by using species-specific primers and ITS primers.

Acknowledgement

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