

Regular Article

Significance of 18S rDNA specific primers in the identification of genus *Dunaliella*

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Abstract

The cells survive in extreme marine environments has received significant interest due to their high valuable compounds. In the present attempt, a total of six different isolates of *Dunaliella* isolated from the salt pans of Andhra Pradesh, India were identified based on their morphology and cultural characteristics. Besides, the isolates were subjected to molecular identification using 18S rDNA specific primers. Out of the six isolate one was never amplified with the any of species specific primers used hence it was partially sequenced and submitted in GenBank. This study obviously describes the incidence of non carotenogenic strains (never turn from green to red) of *Dunaliella bardawil* and *Dunaliella parva* in natural environment.

Key words: *Dunaliella*, salt pans, 18S rDNA, non carotenogenic

Introduction

The genus *Dunaliella* is the physiologically best studied unicellular green algae recognized as being the only eukaryotic and photosynthetic organisms able to grow in an extremely wide range of salt concentrations, from 0.5 M to saturation 5 M. In contrast to most other green algae, cells of the genus *Dunaliella* lack a rigid cell wall, which is enclosed solely by a thin elastic plasma membrane, even though which inhabits hyper saline environments by the production of glycerol by maintaining osmosis. *Dunaliella* are often orange-red in color due to a massive accumulation of β -carotene, a property that makes these algal strains an excellent model for elucidation of the carotenoids biosynthetic pathway and its regulation in photosynthetic organisms (Lers *et al.*, 1990; Shaish *et al.*, 1991, 1992).

Since the description of *D. salina*, the type species of the genus, by Teodoresco in 1905, approximately 28 species have been recognized, 16 of which were described by Massyuk (1973a, b, c). In her monograph, Massyuk combined morphological and structural features (species level) with some physiological and biochemical characteristics to divide the genus into two subgenera, *Pascheria* and *Dunaliella*. Within the subgenus *Dunaliella*, the taxonomy at the species level is obscure, and some species described as separate entities may be found eventually to be polymorphic forms of only one taxon (Preisig, 1992). It is well established in the literature that morphological (cell shape and size) and structural attributes (presence or absence of stigma, refractile granules, chloroplast size and shape) are not consistently good descriptors in unicellular greens (Gonzalez *et al.*, 2001). In spite of the excellent work of Massyuk many of the strains in culture collections are misnamed, and some unnecessary species names have arisen (Borowitzka and Borowitzka, 1988). This confusion of names and species makes comparison of results by different authors extremely difficult. *Dunaliella bardawil* and *D. parva* are known to show red orange cells in high salinity as per the Massyuk's description. As the above authors, the present results were also distinguish the morphology and molecular level of the genus *Dunaliella* and markedly identified the new variety of *D. bardawil* and *D. parva*.

Material and Methods

Isolation and growth conditions

A total of 21 salt pan water samples of green, orange and red colours were collected in sterile plastic vials from Andhra Pradesh,

South India were screened for *Dunaliella* under compound microscope. The samples contained *Dunaliella* were transferred to De Walne's medium and kept at $24 \pm 1^\circ\text{C}$ in thermostatically controlled room, illuminated with cool fluorescent lamps at irradiance of $30 \mu\text{Em}^{-2}\text{s}^{-1}$, under 12 h/12 h light/dark photo period. After 10 days the samples were serially diluted up to 10^{-4} and 0.1 mL spread on 2 % De Walne's agar medium. Distinct colonies developed on the plates were picked and transferred to De Walne's medium (Orset and Young, 1999) for further investigation. Six different isolates of *Dunaliella* were isolated from the salt pan samples. The cyanobacterial contaminants were eliminated by treating them with 3000 ppm of the antibiotic, streptomycin sulphate for 30 min under $30 \mu\text{Em}^{-2}\text{s}^{-1}$ light intensity and then transferred to antibiotic free basal medium (Rengasamy *et al.*, 1987). The cultures were made axenic by triple antibiotic treatment as described by Droop (1967).

Morphological identification

A total of 6 isolates of *Dunaliella* successfully isolated and maintained in the basal medium under laboratory conditions were segregated based on their morphological characteristics viz., cell shape, cell colour, cell length (L), width (W), flagella length (F) chloroplast arrangement, and growth conditions. The mean cell length and breadth of the cells were calculated from the measurements of 100 cells. They were identified and designated as *D. maritima* viz., MUAP 301, MUAP 302, MUAP 305, two isolate of each *D. salina* MUAP 303, MUAP 304 and one isolate of *D. bioculata* MUAP 306, (Massyuk, 1973; Avron and Ben-Amotz, 1992; Preisig, 1992; Leonardi and Caceres, 1997). All the isolates were maintained in De Walne's medium. All the isolates were maintained in De Walne's medium.

Growth study

All the isolates of *Dunaliella* were studied for their growth under laboratory conditions. Ten mL of optimally grown cultures of *Dunaliella* were inoculated in 90 mL of basal De Walne's medium and kept under the laboratory conditions. This experiment was conducted for a period of 30 days. At every 3 days interval the following parameters viz., i) Cell number (Neubauer haemocytometer) (Cn) and ii) concentrations of pigments viz., Chlorophyll a (Chl a), Chlorophyll b (Chl b) and Total carotenoids (Tc) ($\mu\text{g/mL}$) were recorded (Lichtenthaler, 1987). In addition, division rates were calculated during the exponential phase (Guillard, 1973). Growth curves were plotted against days and \log_{10} of cell number. All the experiments were carried out in triplicates. The mean values are presented in the paper.

Molecular identification

All the 6 isolates of *Dunaliella* were subjected for molecular identification in order to ascertain their systematic position. The genomic DNA of the isolates was isolated according to Sambrook *et al.* (1989). Further they were subjected to amplification with five sets of primers i.e. 2 genus specific and 3 species specific primers for crisscross analysis. The Analytical grade chemicals were used for this purpose.

PCR amplification

The oligonucleotides, MA1 [5' CGG GAT CCG TAG TCA TAT GCT TGT CTC 3'] MA2 [5' CGG AAT TCC TTC TGC AGG TTC ACC 3'] MA3 [5' GGA ATT CCG GAA ACC TTG TTA CGAC 3'] are well conserved

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among the genus, strains of *Dunaliella* and used the following combinations MA1-MA2 and MA1-MA3. The species specific primers such as *Dunaliella salina* (DSs) [5' GCA GGA GAG CTA ATA GGA 3'] *Dunaliella bardawil* (DBs) [5' GGG AGT CTT TTT CCA CCT 3'] *Dunaliella parva* (DPs) [5' GTA GAG GGT AGG AGA AGT 3'] were also used. The species specific primers were used in combination with MA2 as a reverse primer described by Olmos et al. (2000, 2002). PCR product was resolved on 1.4% agarose gel along with a 500 bp marker Genei, Bangalore (India). The molecular weight of amplified product was calculated and confirmed using Vilber Loumart gel documentation systems.

Partial sequence of *Dunaliella* isolates

Dunaliella maritima MUAP 305 yet to be confirm through molecular technique chosen for partial sequence. The 18S rDNA regions of *D. maritima* MUAP 305 were amplified with MA1-MA2 primers and the 18S rDNA was partially sequenced using Applied Bio system Instrument (ABI) Prism 310 Genetic and submitted in GenBank.

Results

***Dunaliella maritima* MUAP 301**

Cells oval or ellipsoidal with two smooth equal long flagella inserted apically; chloroplast shifted towards the anterior region, each cells 7.0 – 12.0 µm long, and 7.0 – 12.0 µm wide; flagella 14.0 – 17.0 µm long with radial symmetry (Fig. 1a). This isolate was placed under non carotenogenic group since it did not turn red-orange

phase. This organism showed a maximum growth rate of 6.95 log10 cells/ mL on 24th day and division rate of 1.13 days-1. Maximum concentrations of Chl a, Chl b and total carotenoids of 4.7, 2.1 and 3.6 µg/mL were recorded on 21st, 15th and 30th days, respectively (Fig. 2a).

Fig. 1 Morphology of *Dunaliella* isolates from salt pans of Andhra Pradesh, India under light microscope. (a) *D. maritima* MUAP 301 (b) *D. maritima* MUAP 302 (c) *D. salina* MUAP 303 (d) *D. salina* MUAP 304 (e) *D. maritima* MUAP 305 (f) *D. bioculata* MUAP 306

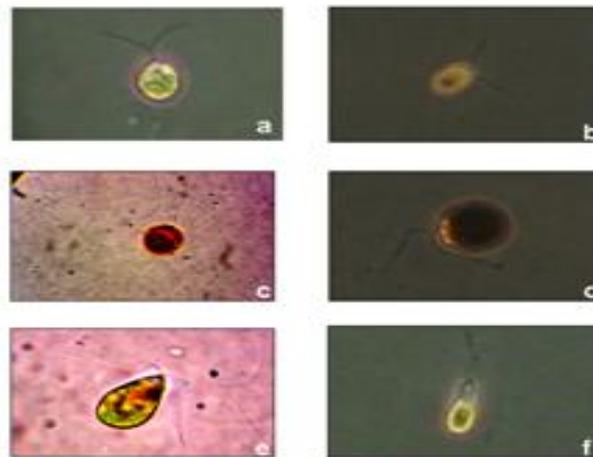
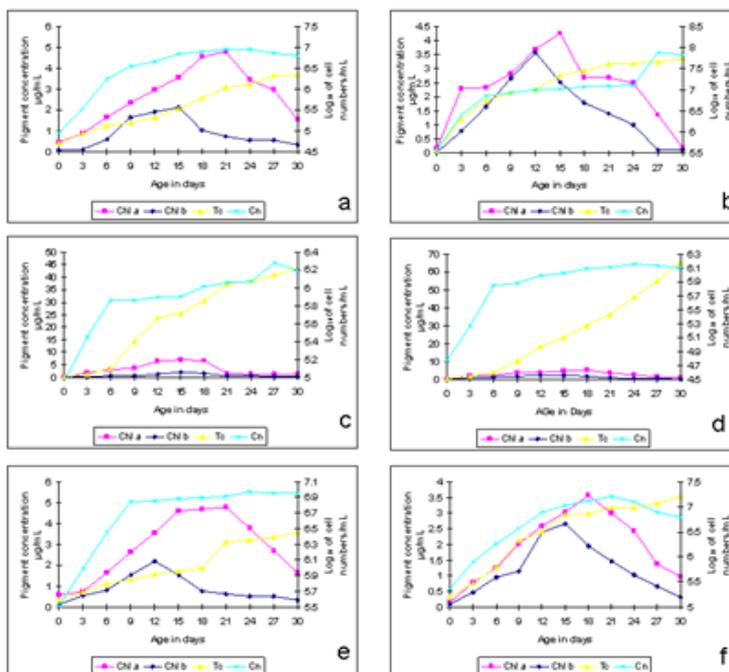


Fig. 2 Growth characteristics and pigment concentrations of *Dunaliella* isolates. (a) *D. maritima* MUAP 301 (b) *D. maritima* MUAP 302 (c) *D. salina* MUAP 303 (d) *D. salina* MUAP 304 (e) *D. maritima* MUAP 305 (f) *D. bioculata* MUAP 306



***Dunaliella maritima* MUAP 302**

Cells oval or ellipsoidal with two smooth equal long flagella inserted apically; chloroplast shifted towards the anterior region, each cells 7.0 – 12.0 µm long, and 7.0 – 12.0µm wide; flagella 14.0 – 17.0 µm long with radial symmetry (Fig. 1b). This isolate was placed under non carotenogenic group since it did not turn red-orange phase. This organism showed a maximum growth rate of 7.88 log10 cells/ mL on 27th day and division rate of 1.109 days-1. Maximum concentrations of Chl a, Chl b and total carotenoids of 4.2, 3.3 and 3.5 µg/mL were recorded on 21st, 12th and 30th days, respectively (Fig. 2b).

***Dunaliella salina* MUAP 303**

Cells oblong, pyriform, ellipsoidal to cylindrical with round anterior and posterior regions with two equal long flagella; chloroplast

situated in the basal region; each cell 11.0 – 13.0 µm long and 9.5 – 11.5 µm wide; flagella 16.0 – 20.0 µm long (Fig. 1c). This isolate was placed under carotenogenic group since it turned to red-orange phase. It showed a maximum growth rate of 6.28 log10 cells/mL on 27th day and division rate of 1.02 days-1. Maximum concentrations of Chl a, Chl b and total carotenoids of 6.8, 2.2 and 43.7 µg/mL were recorded on 15th, 15th and 30th days, respectively (Fig. 2c).

***Dunaliella salina* MUAP 304**

Cells oblong, pyriform, ellipsoidal to cylindrical with round anterior and posterior regions with two equal long flagella; chloroplast situated in the basal region; each cell 15.0 – 17.5 µm long and 10.5 – 13.5 µm wide; flagella 14.0 – 16.5 µm long (Fig. 1d). This isolate was placed under carotenogenic group since it turned to red-orange phase. It showed a maximum growth rate of 6.28 log10 cells/mL on

24th day and division rate of 1.03 days⁻¹. Maximum concentrations of Chl a, Chl b and total carotenoids of 5.0, 2.3 and 65.2 µg/mL were recorded on 18th, 15th and 30th days, respectively (Fig. 2d).

Dunaliella maritima MUAP 305

Cells oval or ellipsoidal with two smooth equal long flagella inserted apically; chloroplast shifted towards the anterior region, each cells 7.0 – 12.0 µm long, and 7.0 – 12.0µm wide; flagella 14.0 – 17.0 µm long with radial symmetry (Fig. 1e). This isolate was placed under non carotenogenic group since it did not turn red-orange phase. This organism showed a maximum growth rate of 6.97 log₁₀ cells/mL on 24th day and division rate of 1.041 days⁻¹. Maximum concentrations of Chl a, Chl b and total carotenoids of 4.7, 2.2 and 3.5 µg/mL were recorded on 21st, 12th and 30th days, respectively (Fig. 2e).

Dunaliella bioculata MUAP 306

Cells always green in colour with posterior broader and anterior narrow regions; cup shaped chloroplast located at the basal region; stigma is seen at the anterior region but not clearly visible. Each cell 10.0 –12.0 µm long and 5.0 – 7.0 µm wide; flagella 12.0 – 14.0 µm long (Fig. 1f). This isolate was placed under non carotenogenic group (did not turn to red-orange phase). The organism showed maximum growth rate of 7.21 log₁₀ cells/mL on 24th day and a division rate of 1.10 days⁻¹. Maximum concentrations of Chl a, Chl b and total carotenoids of 3.5, 2.6, 3.5 µg/mL were recorded on 18th, 15th and 30th days, respectively (Fig. 2f).

Molecular identification of Dunaliella

All the 6 isolates of *Dunaliella* were subjected for amplification using 18S rDNA regions. Table 1 shows a comparison of identification of the *Dunaliella* isolates based on their morphological characteristics as well as by molecular tools. The amplified products of 18S rDNA by using MA1-MA2 primers, identified morphological characteristics namely, *D. maritima* MUAP 301, MUAP 302, MUAP 305, *D. bioculata* MUAP 306 showed ca. 2570 bp, whereas with MA1-MA3 primers exhibited ca. 2170 bp. The amplified products of *D. salina* MUAP 303, MUAP 304 showed ca.1700 bp when the primers of both MA1-MA2 and MA1-MA3 primer pairs were used (Figs. 3a, 3b, Table 1). When the 6 isolates amplified with the three species specific primers, isolates such as *D. salina* MUAP 303 and *D. salina* MUAP 304 were amplified a product of ca. 700 bp, thus confirmed their identity as *D. salina* (Fig. 3c, Table 1). The 18S rDNA region of *D. bioculata* MUAP306 were amplified with the species specific DBs primer (ca. 1000 bp) and therefore it was assigned to *D. bardawil* (Fig. 3d, Table 1). The two isolates viz., *D. maritima* MUAP 301, MUAP 302 were amplified with the DPs primer (ca.1000 bp) and confirmed their identity as *D. parva* (Fig. 3e, Table 1). The 18S rDNA regions of the isolate *D. maritima* MUAP 305 did not be amplify with any one of the species specific primers such as DSs, DBs and DPs used. Therefore, they do not belong to *D. salina*, *D. bardawil* and *D. parva* (Figs. 3c, 3d, 3e, Table 1). The 18S rDNA region of *D. maritima* MUAP 305 was subjected for partial sequence and their length of about 1 – 386 bp, the sequence was submitted in the GenBank, NCBI and the accession number is GU454803.

Fig. 3 (a) Amplification of morphological identified isolates using MA1 and MA2 primers
 Lane 1: 500 bp marker Lane 2: *D. maritima* MUAP 301
 Lane 3: *D. maritima* MUAP 302 Lane 4: *D. salina* MUAP 303
 Lane 5: *D. salina* MUAP 304 Lane 6: *D. maritima* MUAP 305
 Lane 7: *D. bioculata* MUAP 306 Lane 7: Negative control
 (b) Amplification of morphological identified isolates using MA1 and MA3 primers
 Lane 1: 500 bp marker Lane 2: *D. maritima* MUAP 301
 Lane 3: *D. maritima* MUAP 302 Lane 4: *D. salina* MUAP 303
 Lane 5: *D. salina* MUAP 304 Lane 6: *D. maritima* MUAP 305
 Lane 7: *D. bioculata* MUAP 306 Lane 7: Negative control
 (c) Amplification of morphological identified isolates using DSs and MA2 primers
 Lane 1: 500 bp marker Lane 2: *D. maritima* MUAP 301
 Lane 3: *D. maritima* MUAP 302 Lane 4: *D. salina* MUAP 303
 Lane 5: *D. salina* MUAP 304 Lane 6: *D. maritima* MUAP 305
 Lane 7: *D. bioculata* MUAP 306 Lane 7: Negative control
 (d) Amplification of morphological identified isolates using DBs and MA2 primers
 Lane 1: 500 bp marker Lane 2: *D. maritima* MUAP 301
 Lane 3: *D. maritima* MUAP 302 Lane 4: *D. salina* MUAP 303
 Lane 5: *D. salina* MUAP 304 Lane 6: *D. maritima* MUAP 305
 Lane 7: *D. bioculata* MUAP 306 Lane 7: Negative control
 (e) Amplification of morphological identified isolates using DPs and MA2 primers
 Lane 1: 500 bp marker Lane 2: *D. maritima* MUAP 301
 Lane 3: *D. maritima* MUAP 302 Lane 4: *D. salina* MUAP 303
 Lane 5: *D. salina* MUAP 304 Lane 6: *D. maritima* MUAP 305
 Lane 7: *D. bioculata* MUAP 306 Lane 7: Negative control

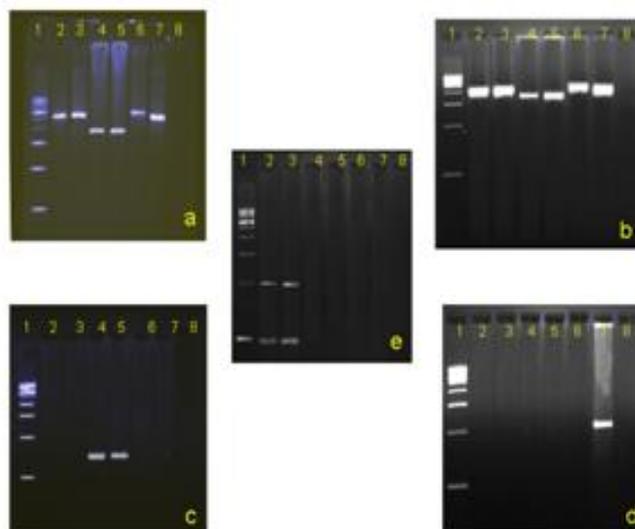


Table 1 Amplified product size of *Dunaliella* isolates using different primer pairs MA1- MA2, MA1- MA3, and with species specific primers DSs - MA2, DBs - MA2, DPs -MA2

Isolates	Morphological identification	PCR products size of MA1-MA2 (~ bp)	PCR products size of MA1-MA3 (~bp)	PCR products size of DSs-MA2 (~bp)	PCR products size of DBs-MA2 (~bp)	PCR products size of DPs-MA2 (~bp)	Molecular identification
MUAP 301	<i>D. maritima</i>	2570	2170	*	*	1000	<i>D. parva</i>
MUAP 302	<i>D. maritima</i>	2570	2170	*	*	1000	<i>D. parva</i>
MUAP 303	<i>D. salina</i>	1700	1700	700	*	*	<i>D. salina</i>
MUAP 304	<i>D. salina</i>	1700	1700	700	*	*	<i>D. salina</i>
MUAP 305	<i>D. maritima</i>	2570	2170	*	*	*	<i>Dunaliella</i> sp.
MUAP 306	<i>D. bioculata</i>	2570	2170	*	1000	*	<i>D. bardawil</i>

Discussion

Among the algae, *Dunaliella* occurs mostly in hypersaline environments (Ben Amotz et al., 1982; Borowitzka and Borowitzka, 1988; Herrero et al., 2006; Hu et al., 2008). In the present study, among the 6 different isolates of *Dunaliella* isolated from Andhra Pradesh salt pans two isolates such as *D. salina* MUAP 303, MUAP 304 showed red orange carotenogenic phase on 18th day. Whereas the rest of isolates identified through morphological and cultural characteristics viz., *D. maritima* MUAP 301, MUAP 302, MUAP 305 and *D. bioculata* MUAP 306 never turn to orange phase and hence,

they were considered as non carotenogenic group. All the isolates exhibited significant variations in size, shape and division rates. The cells of *Dunaliella salina* MUAP 303 were larger in size and showed lesser division rates when compared to other isolates, which were smaller in size. The results was in accordance with Cifuentes et al. (1992) inferred that the size and rate of cell division were inversely proportional. Levels of the three pigments viz., Chl a, Chl b and total carotenoids were investigated in all the isolates and obviously the carotenogenic isolate contained very high level of total carotenoids. Maximum accumulation of total carotenoids was recorded in *D. salina* MUAP 304 (65.2 µg/mL) on 30th day. Maximum concentration

of Chl a was recorded in *D. salina* MUAP 303 of 6.8 µg/mL on 15th day, the same for Chl b concentrations was observed in *D. maritima* MUAP 302 on 12th day. The concentrations of Chl a and Chl b gradually increased up to 15th and 18th day, respectively, followed by marginal decrease in their concentrations. In spite of the absence of any growth by cell number after 24/27 days, the concentration of total carotenoids steadily increased up to 30 days. The observations indicated that the pigments such as Chl a and Chl b were predominant in the growth phase of *Dunaliella* while the amount of total carotenoids increased and accumulated to high levels in carotenogenic alga during the red orange phase. The continuance in the increase of carotenoids observed in the present study in the algal isolates even after cessation of growth i.e. between 24-30 days could also be attributed to nutrient depleted conditions (Ben-Amotz et al., 1987; Borowitzka and Borowitzka, 1989; Vorst et al., 1994; Shelly et al., 2002). Identification of the 6 isolates of *Dunaliella* through molecular techniques revealed certain interesting findings and they are discussed as follows. Two primer pairs such as i) MA1-MA2, ii) MA1-MA3, meant for genus specific and three primers such as iii) DSs-MA2, iv) DBs-Ma2, and v) DPs-MA2 for species specific were used in the present investigation. All the 6 isolates were subjected for amplification with all five sets of primers for crisscross analysis. The amplified products of MA1-MA2 primers were at the generic level and the MA1-MA3 at strain level (Olmos et al., 2000). In the present attempt, the two sets of primers mentioned above showed good amplification with all the isolates. The amplified products of 18S rDNA by using MA1-MA2 on the following isolates identified through morphological and cultural features of *D. maritima* MUAP 301, MUAP 302, MUAP 305, *D. bioculata* MUAP 306 showed ca. 2570 bp, whereas with MA1-MA3 primers, showed ca. 2170 bp. The amplified product of *D. salina* MUAP 303, MUAP 304 showed ca. 1700 bp when both MA1-MA2 and MA1-MA3 primers were used in conjunction. Therefore, *Dunaliella* showed three different kinds of products i.e. i) 2570 bp, ii) 2170 bp, and iii) 1700 bp when subjected to MA1-MA2 and MA1-MA3 primers thus suggested that 18S rDNA of the isolates are well conserved. The amplified products of 2570 bp indicated that they possessed two introns when the MA1-MA2 primers were used. However, with MA1-MA3 primers, the products size 2170 bp for the isolates while the number of introns remained the same. The product with ca. 1700 bp indicated that there was without introns. The above results are in accordance with the observations made by Olmos et al. (2000) and Raja et al. (2007). The PCR products of *D. bardawil* also were of 2570 bp and 2170 with the primers MA1-MA2 and MA1-MA3, respectively, indicating the presence of two introns (Wilcox et al. 1992; Olmos et al. 2000). The observations made in the present study as well as Olmos et al. (2000) showed the distinctive nature of the 18S rDNA in different species of *Dunaliella*.

To achieve concordant results, all the isolates were separately run with three species specific primers to confirm their identity. For example *D. salina* MUAP 303 showed amplification of 18S rDNA region with DSs primer but not with the other two primers, DBs and DPs. Therefore it was confirmed that the identification made in the present study was properly carried out. All the amplified products showed single band on the gel. The DSs-MA2 primers gave a length of approximately 700 bp and DBs and DPs gave 1000 bp product. Alternatively, a band of 500 bp amplified due to DPs had another specific site of binding, which has not interfered with any of the results obtained. These observations matched with that of Olmos et al. (2000). Among the isolates, only the isolates MUAP 303 and MUAP 304 identified as *D. salina* through morphological and cultural characteristics were similar to the identification made through molecular tools. Surprisingly identification of the remaining isolates through morphological and cultural characteristics did not match to that of molecular tools. The isolate *D. bioculata* MUAP 306 identified through morphological and cultural characteristics were assigned to *D. bardawil* based on molecular markers. Similarly *D. maritima* MUAP 301 and *D. maritima* MUAP 302 were identified as *D. parva*.

Nevertheless, confusion still prevailed in the present study with respect to the grouping of the alga into carotenogenic and non-carotenogenic, since some of the non carotenogenic isolates i.e. which do not have the ability to turn to carotenogenic phase contained less production of total carotenoids. *Dunaliella salina*, *D. bardawil* and *D. parva* are known to turn reddish orange in medium

(Ben Amotz et al., 1982; Shaish et al., 1991; Jimenez and Pick, 1994; Orset and Young, 1999). However, in our investigation the isolates such as *D. parva* MUAP 301, MUAP 302, *Dunaliella* sp. MUAP 305 and *D. bardawil* MUAP306 identified through molecular tools never turned to carotenogenic phase (reddish orange) through out the study period. Thus, it is evident that of *D. bardawil* and *D. parva* are distinctly different from that reported by Ben Amotz et al. (1982), Shaish et al. (1991), Jimenez and Pick (1994), Orset and Young (1999) and therefore the present study revealed that isolates of *D. bardawil* and *D. parva* are pale yellowish green in culture condition with poor production of total carotenoids which clearly indicates the presence of non carotenogenic or poor carotenogenic strains may also exist in natural environment. Polle et al. (2008) reported that the cells of *D. salina* did not turn bright orange, suggesting that cellular levels of β-carotene in the Korean strain were much low as compared to that in other strains.

The 18S rDNA of the isolate *D. maritima* MUAP 305 identified through morphological and cultural features did not amplify using any of the three different species specific primers such as DSs, DBs, and DPs used and therefore they do not belong to *D. salina*, *D. bardawil* and *D. parva*. Therefore the systematic position of the above one isolate remained to be confirmed through molecular tools. As per the investigation the above one isolate belong to genus *Dunaliella* but they were not identified their species level, so the isolate was subjected for sequencing, even after that the species level could not be revealed with the present database, so it was named as (*Dunaliella* sp.).

In the present study, the isolates of *Dunaliella* exhibited different shapes such as oblong, spherical, round and sometimes pyriform and therefore it is a hard task to identify them through classical approaches which considered the shape of the alga as one of the criteria for identification. The names associated with the many strains of *Dunaliella* in culture collections are often clearly incorrect and the origin and history of several of the strains is confused. The systematic application of molecular methods as well as cladistic analysis will be important in developing a better understanding of the taxonomy, systematic and phylogeny of this genus, but they also present new challenges (Borowitzka and Siva, 2007). The present investigation, evidently provoke that both the classical and molecular tools should club together to present a distinct identification in the *Dunaliella* genus.

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