ISSN: 0975-833X

Available online at http://www.journalcra.com

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

International Journal of Current Research
Vol.8, pp.073-078, September, 2010

RESEARCH ARTICLE

DISCRIMINATION BETWEEN THE MORPHOLOGICAL AND MOLECULAR IDENTIFICATION IN THE GENUS *DUNALIELLA*

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ARTICLE INFO

Article History:
Received 11th July, 2010
Received in revised form
24th July, 2010
Accepted 25th August, 2010
Published online 4th September, 2010

Key words:
Dunaliella , Salt pans,
Gen bank, β-carotene and Andhra Pradesh

ABSTRACT

Six different isolates of *Dunaliella* were isolated along the salt pans of Andhra Pradesh coast, India were focused by classical and molecular taxonomy which effectively reported the significance of molecular tools and identified the strange green forms of *Dunaliella bardawil* and *Dunaliella parva*. Among the six isolates five were identified with present genus and species specific primers used in the study where as the remaining one isolate was partially sequenced and submitted in the Gen bank.

INTRODUCTION

Dunaliella is a unicellular, ovoid, biflagellate, naked green alga. The cells are motile and have two equal, long smooth whiplash flagella which belong to the Order Volvacales, Family Polyblepharidaceae and the class of Chlorophyceae. It was first identified by a French Scientist Michel Felix Dunal in 1838 later it was discovered by Teodoresco in 1905. The unique morphological feature of *Dunaliella* is that lacks a cell wall. The cell is enclosed by a thin plasma membrane or periplast, which permits rapid changes in cell shape and volume in response to osmotic changes. To survive, these organisms have high concentrations of β-carotene to protect against the intense light and high concentrations of glycerol to provide protection against osmotic pressure. Twenty eight species of *Dunaliella* are presently recognized.

Few organisms can survive in such highly saline conditions as salt evaporation ponds that accumulate massive amounts of carotenoids under appropriate growth conditions. Natural mixed carotenoids obtained from *Dunaliella* contain both 9-cis and all-trans isomers. All-trans isomers exhibit Vitamin A activity and 9-cis provides potent antioxidant activity, unlike synthetic beta-carotene which contains only trans isomers. From a first pilot plant for *Dunaliella* cultivation for β-carotene protection established in the USSR in 1966, the commercial cultivation of *Dunaliella* for the production of beta carotene throughout the world is now one of the success stories of halophile biotechnology.

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Research performed at the Cancer Research Centre at Hawaii showed that *Dunaliella* contains a certain type of beta-carotene called 9-cis-beta-carotene, which is up to ten times stronger at preventing cancer than ordinary beta-carotene (Hieber *et al.,* 2000).

The cell morphology depends largely on the environmental factor such as salinity, light intensity, and age of the culture. Because of great morphological variations even within the one species, there has been confusion as to the correct classification various described species and sub species only recently as addition molecular analyses various techniques been used to further study the phylogeny of genus *Dunaliella.* (Jin and Polle, 2006). Species specific oligonucleotide could be useful to identify both the strains from culture collection and from natural environment. Slight morphological difference in *Dunaliella* species can conceal profound differences in their potential production of metabolites such as carotenoids. Conserved and variable regions of 16S -18S r DNA sequences have been used as target for framer-directed DNA amplification by polymerase chain reaction (PCR) for the identification of microorganisms. (Olsen *et al.,* 1986, Jeyarao *et al.,* 1991). The development of modern biotechnological tools such as Polymerase Chain Reaction and analysis of DNA/ rRNA based technology ascertain the identification the micro organism. In the present study, six different isolates of *Dunaliella* isolated from the salt pans of Andhra Pradesh coast, India were chosen for identification by both morphological and molecular aspects and enroll the significance of molecular taxonomy.

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±1 °C in thermostatically controlled room, illuminated with cool fluorescent lamps at irradiance of 30 $\mu Em^{-2}s^{-1}$, 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 minutes under 30 $\mu Em^{-2}s^{-1}$ 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 *Dunaliella bioculata*, MUAP 101, MUAP 102, and one isolate of each *Dunaliella tertiolecta* MUAP 103, *Dunaliella viridis* MUAP 104*, Dunaliella minuta* MUAP 105 and *Dunaliella maritima* MUAP 106 (Massyuk, 1973 a, b, c; 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. They were also subjected to different NaCl concentration ranging from 0.5 M to 5 M and studied for their growth. Out of the salinities tested all the isolates showed optimal growth at 2.14 M NaCl salinity (more than 12%).

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 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 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 tertiolecta MUAP 103 yet to be confirm through molecular technique chosen for partial sequence. The 18S rDNA regions of *D. tertiolecta* MUAP 103 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 Gen bank.

RESULTS

Morphological identification

Dunaliella bioculata MUAP 101

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.1a).

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	Amplified products size of MA1-MA2 (~ bp)	Amplified products size of MA1-MA3 (~bp)	Amplified products size of DSs-MA2 (~bp)	Amplified products size of DBs-MA2 (~bp)	Amplified products size of DPs-MA2 (~bp)	Molecular identification
MUAP 101	*D. bioculata*	2570	2170	*	*	1000	*D. parva*
MUAP 102	*D. bioculata*	2570	2170	*	1000	*	*D. bardawil*
MUAP 103	*D. tertiolecta*	2570	2170	*	*	*	*Dunaliella* sp.
MUAP 104	*D. viridis*	2570	2170	*	*	1000	*D. parva*
MUAP 105	*D. minuta*	2570	2170	*	*	1000	*D. parva*
MUAP 106	*D. maritima*	2570	2170	*	*	1000	*D. parva*

* Amplification not observed

This isolate was placed under non carotenogenic group (did not turn to red-orange phase).

***Dunaliella bioculata* MUAP 102**

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. 1b). This isolate was placed under non carotenogenic group (did not turn to red-orange phase).

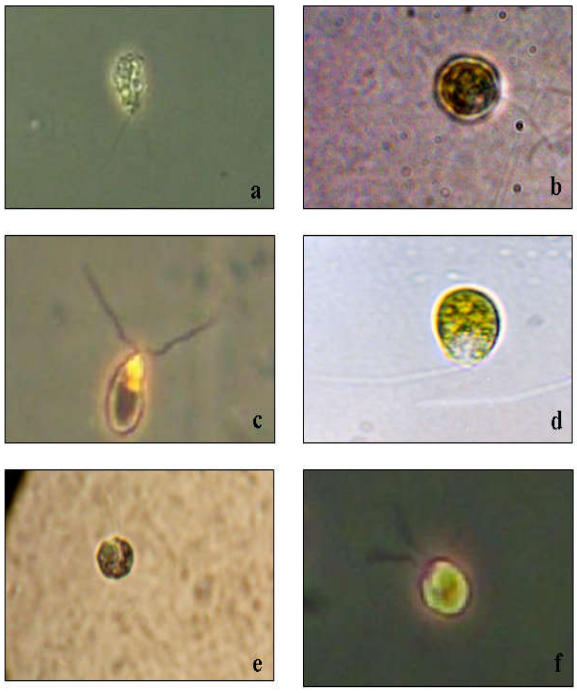

Fig. 1. Morphology of *Dunaliella* isolates from salt pans of Andhra Pradesh, India under light microscope. (a) *D. bioculata* MUAP 101 (b) *D. bioculata* MUAP 102 (c) *D. tertiolecta* MUAP 103 (d) *D. viridis* MUAP 104 (e) *D. minuta* MUAP 105 (f) *D. maritima* MUAP 106

***Dunaliella tertiolecta* MUAP 103**

Cells always green, ellipsoidal, oval, pyriforms, apically broader and posterior narrow regions, chloroplast located at the basal region; stigma is not clearly visible; each cell 9.5 – 12.0 µm long and 5.0 – 7.0 µm wide; flagella 12.0 – 14.0 µm long (Fig. 1c) with radially symmetrical. This isolate was placed under non carotenogenic group (did not turn to red-orange phase).

***Dunaliella viridis* MUAP 104**

Cells always green, pyriform, ellipsoid, oval or spherical or globular, radially symmetrical, chloroplast located at the basal region; cells 9.5 – 12.0 µm long and 7.0 – 12.0 µm wide; flagella 12.0 – 14.0 µm long (Fig.1d). This isolate was placed under non carotenogenic group (did not turn to red-orange phase).

***Dunaliella minuta* MUAP 105**

Cells always green, cylindrical, oval, elliptical or pyriform with rounded anterior and posterior ends, with two long smooth whiplash flagella 15.0 µm long. Cells 3–13 µm long, 1.5–10 µm wide. Flagella length slightly longer than cell length. Cup shaped chloroplast with lobes nearly at the anterior end of the cell. Eye spot is not clearly visible

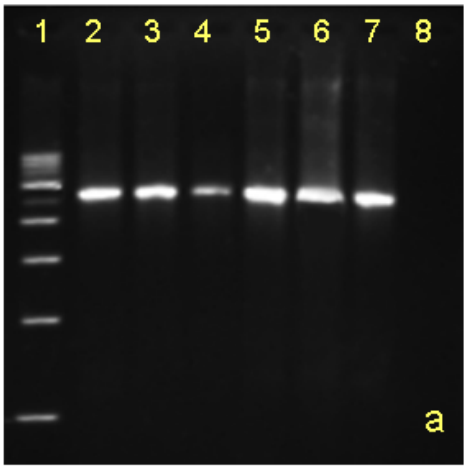

Fig. 2. (a) Amplification of morphological identified isolates using MA1 and MA2 primers

Lane 1: 500 bp marker
Lane 2: *D. bioculata* MUAP 101
Lane 3: *D. bioculata* MUAP102
Lane 4: *D. tertiolecta* MUAP 103
Lane 5: *D. viridis* MUAP 104
Lane 6: *D. minuta* MUAP 105
Lane 7: *D. Maritima* MUAP106
Lane 7: Negative control

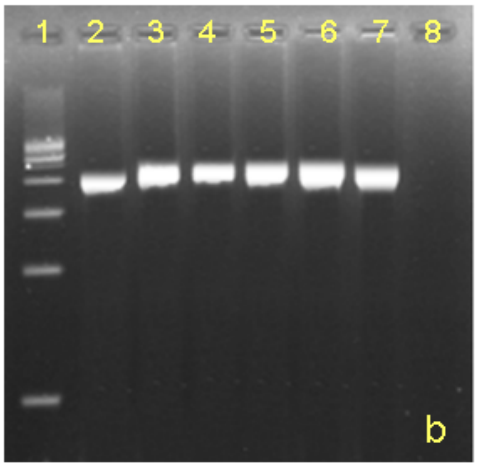

Fig. 2. (b) Amplification of morphological identified isolates using MA1 and MA3 primers

Lane 1: 500 bp marker
Lane 2: *D. bioculata* MUAP 101
Lane 3: *D. bioculata* MUAP102
Lane 4: *D. tertiolecta* MUAP 103
Lane 5: *D. viridis* MUAP 104
Lane 6: *D. minuta* MUAP 105
Lane 7: *D. Maritima* MUAP106
Lane 7: Negative control

(Fig.1e). This isolate was placed under non carotenogenic group since it did not turn to red-orange phase.

***Dunaliella maritima* MUAP 106**

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.1f). This isolate was placed under non carotenogenic group since it did not turn red-orange phase.

Molecular identification of *Dunaliella*

All the 6 isolates of *Dunaliella* were subjected for amplificationWilcox using 18S rDNA regions. Conserved

oligonucleotides complementary to 5' and 3' termini of the 18S rDNA region were used for the characterization. Table 1 shows a comparison of identification of the

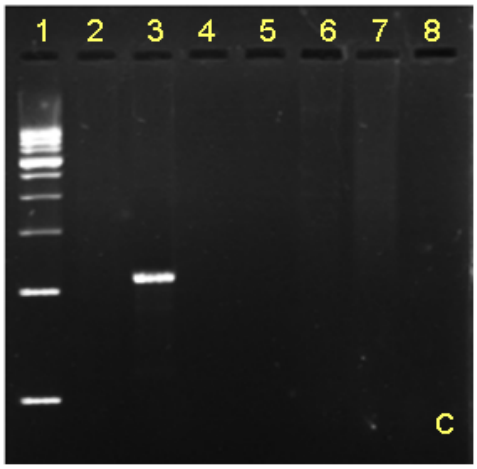

Fig. 2. (c) Amplification of morphological identified isolates using DBs and MA2 primers

Lane 1: 500 bp marker
Lane 2: *D. bioculata* MUAP 101
Lane 3: *D. bioculata* MUAP102
Lane 4: *D. tertiolecta* MUAP 103
Lane 5: *D. viridis* MUAP 104
Lane 6: *D. minuta* MUAP 105
Lane 7: *D. Maritima* MUAP106
Lane 7: Negative control

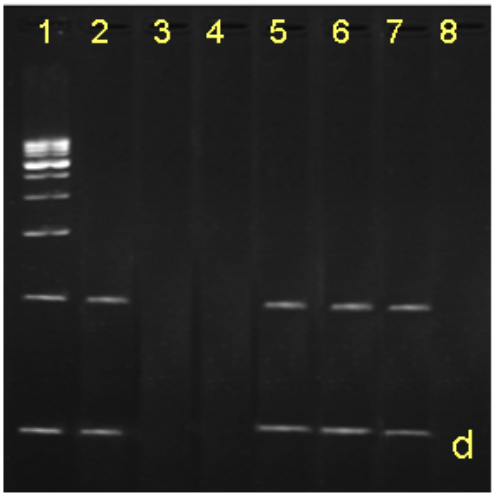

Fig. 2. (d) Amplification of morphological identified isolates using DPs and MA2 primers

Lane 1: 500 bp marker
Lane 2: *D. bioculata* MUAP 101
Lane 3: *D. bioculata* MUAP102
Lane 4: *D. tertiolecta* MUAP 103
Lane 5: *D. viridis* MUAP 104
Lane 6: *D. minuta* MUAP 105
Lane 7: *D. Maritima* MUAP106
Lane 7: Negative control

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 through morphological and culture characteristics namely, *D. bioculata* MUAP 101, MUAP 102, *D. tertiolecta* MUAP 103, *D. viridis* MUAP 104, *D. minuta* MUAP 105 and *D. maritima* MUAP 106 showed ca. 2570 bp (Fig. 2a), whereas with MA1-MA3 primers exhibited ca. 2170 bp (Fig. 2b). Further, all the isolates were subjected for the amplification of 18S rDNA by using all the three species specific primers *viz*., DSs for *Dunaliella salina,* DBs for *D. bardawil* and DPs for *D. parva* as forward primers and MA2 as reverse primer (Olmos *et al.,* 2000). Among the 6 isolates, The 18S rDNA region of *D. bioculata* MUAP102 identified based on their morphological and cultural characteristics were amplified with the species specific DBs primer (ca. 1000 bp) and therefore it was assigned to *D. bardawil* (Fig. 2c;Table 1). The four isolates *viz*., *D. bioculata* MUAP 101, *D. viridis* MUAP 104, *D. minuta* MUAP 105 and *D. maritima* MUAP 106 were amplified with the DPs primer (ca.1000 bp) and confirmed their identity as *D. parva* (Fig. 2d; Table1). The 18S rDNA regions of the isolate *D. tertiolecta* MUAP 103 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. 2c, 2d; Table 1). The 18S rDNA region of *D. tertiolecta* MUAP 103 was subjected for partial sequence and their length of about 1 – 423 bp. The sequence was submitted in the Genbank, NCBI and the accession number is GU454804.

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, All the 6 different isolates of *Dunaliella* isolated from Andhra Pradesh salt pans identified through morphological and cultural characteristics were never turn to orange phase and hence, they were considered as non carotenogenic group. Salinity tolerance and accumulation of large amount of β-carotene in the chloroplast have been used to differentiate the sections within the genus (Massyuk, 1973; Preisig, 1992). But our all the isolates of *Dunaliella* were luxuriously grown at De Walne's medium (2.14 M NaCl) but withstand the higher salinity up to 5 M since it may be adapted in hyper saline environments. Based upon the above fact salinity may not be considered to distinguish species of *Dunaliella*. Influence of salinity on carotenoid biosynthesis and growth pattern in *Dunaliella* shows different characters in different media (Fazeli *et al.,* 2006).

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. bioculata* MUAP 101, MUAP 102, *D. tertiolecta* MUAP 103, *D. viridis* MUAP 104, *D. minuta* MUAP 105 and *D. maritima* MUAP 106 showed ca. 2570 bp, whereas with MA1-MA3 primers, showed ca. 2170 bp. Therefore, *Dunaliella* showed two different kinds of products i.e. i) 2570 bp, ii) 2170 bp, and 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 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 (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. bioculata* MUAP 102 showed amplification of 18S rDNA region with DBs primer but not with the other two primers, DSs 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). Surprisingly identification of the remaining isolates through morphological and cultural characteristics did not match to that of molecular tools. The isolate *D. bioculata* MUAP 102 identified through morphological and cultural characteristics were assigned to *D. bardawil* based on molecular markers. Similarly *D. bioculata* MUAP 101, *D. viridis* MUAP 104*, D. minuta* MUAP 105 and *D. maritima* MUAP 106 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.,* 1992; Jimenez and Pick, 1994; Orset and Young, 1999). However, in our investigation the isolates such as *D. parva* MUAP 101, MUAP 104, MUAP 105, MUAP 106, *Dunaliella* sp. MUAP 103 and *D. bardawil* MUAP102 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. tertiolecta* MUAP 103 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 not identified their species level, so the one isolates 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. instead of giving a incorrect identification.

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 above investigation was in accordance with Melkonian and Preisig (1984). They stated that some of the species recognized by Massyuk (1973) may eventually found to be polymorphic forms of a single taxon. It is very clear that morphological/physiological features of the strains of *Dunaliella* are highly variable and therefore, are unreliable for proper identification of the genus and species. Thus distinguishing a mixed population under natural conditions to the level of genus and species is hard to achieve (Olmos *et al.,* 2000).The cell shape is highly variable because of the absence of a rigid cell wall (Oliveira *et al.,* 1980), and can vary along with cell size depending on culture conditions (Riisgard, 1981). Thus, morphological variability and pronounced ability to adapt to the changes in environmental conditions have introduced high degree of uncertainty in the systematics of the genus, *Dunaliella*. Ultrastructural studies carried out on different species of this genus apparently did not help in the discrimination of taxa at interspecific level (Parra *et al.,* 1990).

Moreover, the taxonomy of *Dunaliella*, particularly the carotenogenic strains, has a history of controversy (Borowitzka and Borowitzka, 1988). For example Borowitzka and Borowitzka (1988) considered that *D. bardawil* (Ben Amotz and Avron, 1983) as a *nomen nudum* of *D. salina* Teod, and that *D. salina* was probably *D. parva*. This confusion of names and the species still remains and makes comparison of results by different authors difficult. Under these circumstances, molecular identification provides a useful tool to distinguish between inter and intra specific morphologically similar species (Olsen *et al.,* 1986; Olmos *et al.,* 2000). 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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