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# First record of a marine microalgal species, *Micractinium singularis* (Trebouxiophyceae) isolated from Janghang Harbor, Korea

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**Abstract:** A eukaryotic microalga was isolated from seawater in Janghang Harbor, Korea and its morphological, molecular, and physiological characteristics were investigated. Due to its simple morphology, no distinctive characters were found by morphological observation, such as light microscope or scanning/transmission electron microscopy (S/TEM). However, molecular phylogenetic evidence inferred from the concatenated small subunit (SSU) 18S rRNA and internal transcribed spacer (ITS) sequence data indicated that the isolate belonged to the newly described *Micractinium singularis*. Furthermore, it was clustered with Antarctic *Micractinium* strains and it also showed a psychrotolerant property, surviving at temperatures as low as 5°C. However, its optimal growth temperatures range from 15°C to 25°C, indicating that this microalga is a mesophile. Additionally, gas chromatography-mass spectrometry (GC/MS) analysis showed that the isolate was rich in nutritionally important omega-3 polyunsaturated fatty acid, and high-performance liquid chromatography analysis (HPLC) revealed that the high-value antioxidant lutein was biosynthesized as an accessory pigment by this microalga, with glucose as the major monosaccharide. Therefore, in this study, a Korean marine *M. singularis* species was discovered, characterized, and described. It was subsequently added to the national culture collections.

**Keywords:** first record, Janghang Harbor, Korean marine microalga, *Micractinium singularis*

## INTRODUCTION

*Micractinium* (Chlorophyta, Trebouxiophyceae, Chlorellales) was first described in 1858 by Fresenius (Luo *et al.* 2006) and this genus is known to have spherical to ovoid cells and possess a parietal and cup-shaped chloroplast with a distinct pyrenoid. Since it shares highly similar morphological features with the genus *Chlorella*, morphological discrimination by the bristle formation in the presence of rotifers such as *Brachionus calyciflorus* was proposed by Luo *et al.* (2006). However, it was also stressed out that the identification and classification based only on the morphological characteristics are not appropriate for the members of chlorellacean genera (Pröschold *et al.* 2010). Hence, Luo *et al.* (2010) proposed a new system of generic separation for these groups based on molecular phylogeny of the combined SSU rRNA and ITS sequences and compensatory base changes (CBC) in helix III of ITS2. These

**Table 1.** Description of the sampling site

| Depth (m) | Temperature (°C) | Salinity (PSU[a]) | Latitude | Longitude |
|---|---|---|---|---|
| 0.3 | 7.7 | 22.5 | 36°00′23.96″N | 126°41′23.52″E |

[a]PSU: practical salinity unit.

approaches have been reliable standard methods for genus differentiation between *Chlorella* and *Micractinium*.

Yet, species-level delimitation within the genus *Micractinium* has been hampered by the lack of available diagnostic morphological keys as well as their plasticity in response to both abiotic and biotic stresses. As a result, there are only 16 taxonomically accepted species even though 25 *Micractinium* species have been described so far (Guiry and Guiry 2019). Currently, no comprehensive taxonomic keys exist which allow for reliable species identification in this group. Consequently, many attempts have been made to find diagnostic morphological characters that can be used for determination of *Micractinium* species in accordance to molecular phylogeny.

In this study, a unicellular microalga belonging to the genus *Micractinium* was isolated from Janghang Harbor in Korea and a pure culture was established. Its phylogenetic position based on the concatenated SSU rRNA and ITS sequence analyses revealed that the isolate was clustered with the recently described Antarctic *M. singularis* strain KSF0094 (Chae *et al.* 2019) and possessed a cold-tolerant property. In conclusion of the results, we report information on the first record of this species in Korea and its morphological, molecular, and chemotaxonomic features.

## MATERIALS AND METHODS

### 1. Sample collection and isolation of microalga

Seawater samples were collected from Janghang Harbor in Sinchang-ri, Janghang-eup, Seocheon-gun, Chungcheongnam-do, Korea in March of 2016. The location and physico-chemical data of the sampling site were given in Table 1. Water samples were filtered on 25 μm mesh net to remove grazing organisms and aliquots (100 μL) of the samples were spread onto 1% (w/v) NaCl (Duksan, Ansan, Korea) BG-11 agar plates (UTEX, Austin, TX, USA) supplemented with 100 μg $mL^{-1}$ imipenem (Sigma-Aldrich, St. Louis, MO, USA) to suppress bacterial growth and generate pure cultures (Hong *et al.* 2015). The plates were then incubated at 10°C in a growth chamber (FLI-2010A, Eyela, Tokyo, Japan) with cool fluorescent light (approximately 30 μmole $m^{-1}$ $s^{-1}$) in a light : dark cycle (16 : 8 h) until microalgal colonies were formed. Single colonies were aseptically streaked onto fresh BG-11 agar plates supplemented with 20 μg $mL^{-1}$ imipenem and this step was repeated until an axenic was produced.

### 2. Morphological identification

A single colony was transferred in 100 mL BG-11 medium and the flasks were autotrophically incubated at 10°C. Well-grown live cells were observed by an upright microscope (Microscope Axio Imager.A2, Carl Zeiss, Göttingen, Germany). The *Brachionus* biotest was performed in order to induce bristle formation in the *Micractinium* isolate according to the method developed by Luo *et al.* (2005). *Brachionus calyciflorus* cysts (TB21) were obtained from MicroBioTests (Gent, Belgium).

For SEM, 10 mL aliquots of cultures at approximately 1,000 cells $mL^{-1}$ were fixed for 10 min in osmium tetroxide ($OsO_4$, Electron Microscopy Sciences, Hatfield, PA, USA) at a final concentration of 2% (v/v). The fixed cells were collected on a 3 μm pore size, polycarbonate membrane filter (Whatman, Kent, UK) and washed three times with distilled water to remove residual media components. The membranes were dehydrated in an ethanol series (Merck, Darmstadt, Germany) and immediately dried using a critical point dryer (CPD 003, Bal-Tec, Balzers, Liechtenstein). The dried filters were mounted on a stub and coated with platinum in a low vacuum sputter coater (FM ACE200, Leica, Wetzlar, Germany). Surface morphology was observed with a field emission scanning electron microscopy (FE-SEM, SUPRA 55VP, Carl Zeiss, Jena, Germany).

For TEM, cells were transferred to a 10 mL tube and fixed in 2.5% (v/v) glutaraldehyde for 2 hrs and the content was concentrated at 1,610 *g* for 10 min in a Vision Centrifuge VS-5500 (Bucheon, Korea). The resulting pellet was subsequently transferred to a 1.5 mL tube and rinsed in 0.2 M sodium cacodylate buffer (Electron Microscopy Sciences) at pH 7.4. After several rinses in 0.2 M sodium caco-

 

**Table 2.** Results from BLAST searches (searched date: 22 December 2019) using the 18S rRNA and ITS sequences of *Micractinium singularis* MM0003

| Marker gene | Accession No. | Length (bp) | Closest match (GenBank accession No.) | Overlap (%) | Sequence similarity (%) |
|---|---|---|---|---|---|
| 18S rRNA | KY655270 | 1,770 | *Micractinium* sp. KNUA034 (KM243325) | 100 | 100.00 |
| ITS | KY655271 | 724 | *Micractinium* sp. KNUA036 (KT883910) | 100 | 97.65 |

dylate buffer, cells were post-fixed for 90 min in 1% (w/v) $OsO_4$ in deionized water. The pellet was then embedded in agar (Duksan). Dehydration was performed in a graded ethanol series (50, 60, 70, 80, 90, and 100% ethanol, followed by two changes in 100% ethanol). The material was embedded in Spurr's resin (Electron Microscopy Sciences). Sections were prepared on an EM UC7 ultramicrotome (Leica) and stained with 3% (w/v) aqueous uranyl acetate (Electron Microscopy Sciences) followed by lead citrate (Electron Microscopy Sciences). The sections were visualized on an H-7650 TEM (Hitachi, Tokyo, Japan) using a voltage of 100 kV.

## 3. Molecular identification

For molecular analysis, genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The primer sets NS1 and NS8 and ITS1 and ITS4 (White *et al.* 1990) were used to amplify the SSU 18S rRNA and ITS region, respectively. Synthesis of the primers employed in this study and the DNA sequencing were conducted at the Macrogen facility (Daejeon, Korea). Phylogenetic analysis was performed with the string sequences of SSU-ITS1-5,8S-ITS created according to Luo *et al.* (2010) using the software package Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 (Kumar *et al.* 2016). The combined data set of the isolate was aligned with those of the 11 chlorellacean microalgae strains based on the previous publications (Hoshina *et al.* 2010; Luo *et al.* 2010; Chae *et al.* 2019) using ClustalW incorporated in MEGA 7.0. package (Kumar *et al.* 2016), and closely related sequences were downloaded from the National Center for Biotechnology Information (NCBI) database, manually trimmed, and aligned with MEGA software using the ClustalW tool. The best-fit nucleotide-substitution model (Kimura 2-parameter + a discrete Gamma distribution with 5 rate categories, K2 + G) was selected using MEGA 7.0 based on Bayesian information criterion. This model was used to build a maximum likelihood (ML) phylogenetic tree with 1,000 bootstrap replicates. *M. conductrix* CCAP211/83 was used as an outgroup. DNA sequences obtained in this study were deposited in the NCBI under accession numbers KY655270 and KY655271 (Table 2). The ITS2 secondary structures were constructed using Mfold (Zuker 2003) and 4SALE (Seibel *et al.* 2006, 2008) according to Chae *et al.* (2019).

## 4. Optimal growth condition determination

A single colony of strain MM0003 from a pure culture was aseptically streaked onto BG-11 agar plates in triplicate and incubated for 14 days. Survival and growth of MM0003 cells maintained at temperatures ranging from 5°C to 35°C (at intervals of 5°C) were examined to determine the optimum culture temperature. Salt tolerance test was conducted at 20°C using BG-11 agar supplemented with 0.0 M, 0.5 M, 1.0 M, 1.5 M, and 2.0 M NaCl (Daejung, Siheung, Korea) respectively.

## 5. GC/MS analysis

The isolates were autotrophically grown in BG-11 medium for 20 days and cells were harvested by centrifugation at 2,063 *g* (1580R, Labogene, Daejeon, Korea) for lipid analysis. Then samples were freeze-dried and pulverized to enhance extraction efficiency. Lipid extraction was performed using the method developed by Breuer *et al.* (2013). The FAME composition was analyzed using a 7890A gas chromatograph equipped with a 5975C mass selective detector (Agilent, Santa Clara, CA, USA) according to our previous publication (Kang *et al.* 2019). Compound identification was performed by matching the mass spectra with those in the Wiley/NBS registry of mass spectral data. The searches with a match value higher than 90% were considered valid.

### 6. Biomass characterization

Freeze-dried biomass samples were pulverized with a mortar and pestle and sieved through ASTM No. 230 mesh (opening = 63 μm). Ultimate analysis was conducted in order to determine the carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) contents using a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan, Italy) in duplicate. Oxygen (O) was calculated by subtracting the sum of percent of C, H, N, S and ash from 100%. Gross calorific value (GCV) was estimated by the following equation developed by Given *et al.* (1986): [GCV = 0.3278C + 1.419H + 0.09257S − 0.1379O + 0.637 (MJ $kg^{-1}$)]. Protein content was calculated from the N content in the ultimate analysis by using the conversion factor of × 6.25 (Mariotti *et al.* 2008).

Proximate analysis was carried out on a DTG-60A thermal analyzer (Shimadzu, Kyoto, Japan). Platinum pans were used to contain 30 mg of α-alumina (α-$Al_2O_3$) powder (Shimadzu) as a reference material and approximately 10 mg of each sample, respectively. Nitrogen (> 99.999%, $N_2$) was supplied as the carrier gas at a rate of 25 mL $min^{-1}$ to protect the microalgae powder from oxidation. Samples were heated from 50°C to 900°C at a rate of 10°C $min^{-1}$. Thermogravimetric analysis (TGA) data were analyzed by ta60 Ver. 2.21 software (Shimadzu).

### 7. HPLC analysis

Pigment extraction was performed using the method developed by Zapata *et al.* (2000). Briefly, freeze-dried microalgal biomass was extracted in 90% HPLC-grade acetone (Daejung) and filtered through a Whatman polytetrafluoroethylene (PTFE) syringe filter with a pore size of 0.2 μm (Whatman, Florham Park, NJ, USA). Samples were then analyzed on an Agilent 1260 Infinity HPLC system (Agilent, Waldbronn, Germany) equipped with a Discovery C18 column (25 cm × 4.6 mm, 5 μm; Supelco, Bellefonte, PA, USA) at 33°C based on the method used in our previous publication (Jang *et al.* 2017). HPLC-grade methanol and ammonium acetate were purchased from Daejung and Fluka (Sigma-Aldrich), respectively. Pigment standards such as β-carotene, chlorophyll (Chl)-*a*, Chl-*b*, lutein, and violaxanthin were obtained from Sigma-Aldrich.

### 8. Carbohydrate analysis

For monosaccharide analysis, 50 mg of freeze-dried biomass samples were hydrolyzed in 2.5 mL 2 N sulfuric acid (Sigma-Aldrich) at 94°C for 3 h. When the reaction tubes were cooled to room temperature, a drop of 40% calcium carbonate (Sigma-Aldrich) was added to the hydrolysates. Samples were filtered through a 0.2 μm PTFE filter (Whatman) and analyzed on a Prominence Modular HPLC system (Shimadzu) with a Sugar-Pak I column (10 μm, 6.5 mm × 300 mm; Waters, Milford, MA, USA) according to our previous publication (Kang *et al.* 2019). All monosaccharide standards (arabinose, fructose, fucose, galactose, glucose, lactose, maltose, mannitol, mannose, rhamnose, ribose, sorbitol, sucrose, and xylose) were obtained from Sigma-Aldrich. Monosaccharide contents in mg $g^{-1}$ dry weight (DW) biomass were quantified by calculating the total peak areas of each monosaccharide derived from a calibration curve.

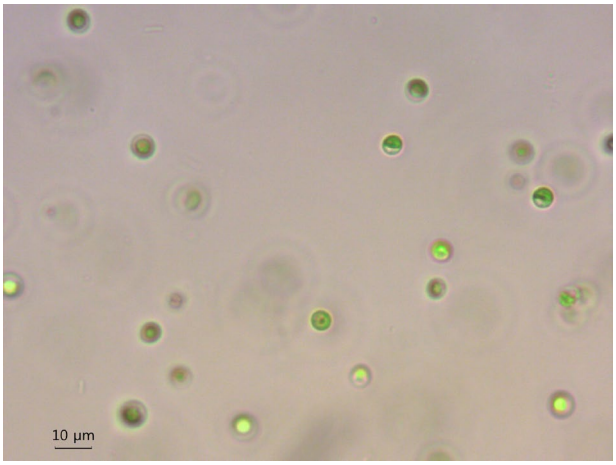


**Fig. 1.** Light micrograph of *M. singularis* MM0003.

## RESULTS

### 1. Morphology of the isolate

As shown in Fig. 1 and Fig. 2, the algal cells were solitary and round to slightly ellipsoid in shape and their sizes ranged from approximately 3 to 5 μm in diameter. No bristles were observed in the isolate in the presence of *B. calyciflorus*. Cytological observation showed that the cells had a predominant cup-shaped chloroplast (C) containing one pyrenoid (P) and multiple layers of thylakoids (Fig. 3). The nucleus (N) was also observed located in the center of the microalga. Starch (S) and mitochondrion (M) were also found in the cells (Fig. 3).

 

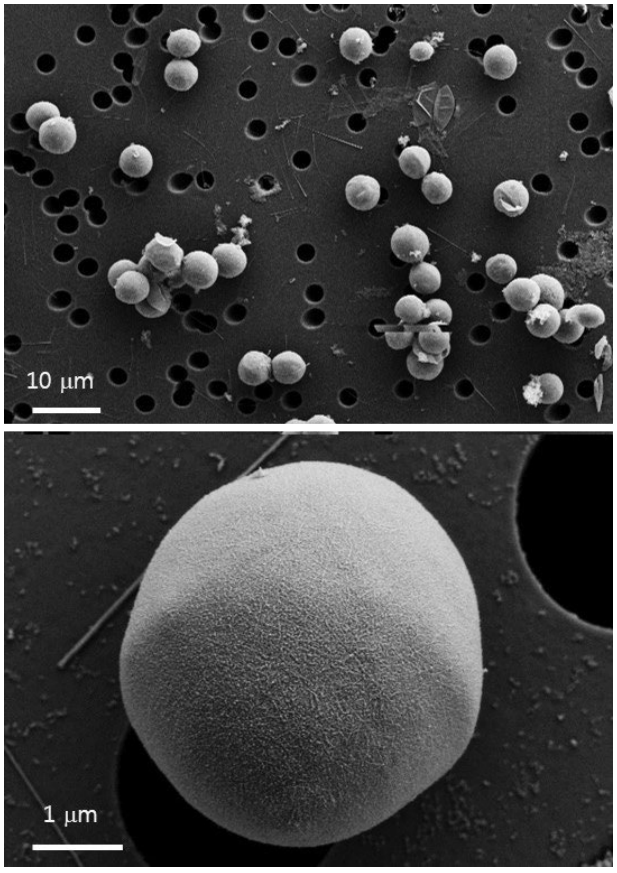


**Fig. 2.** FE-SEM images of *M. singularis* MM0003.

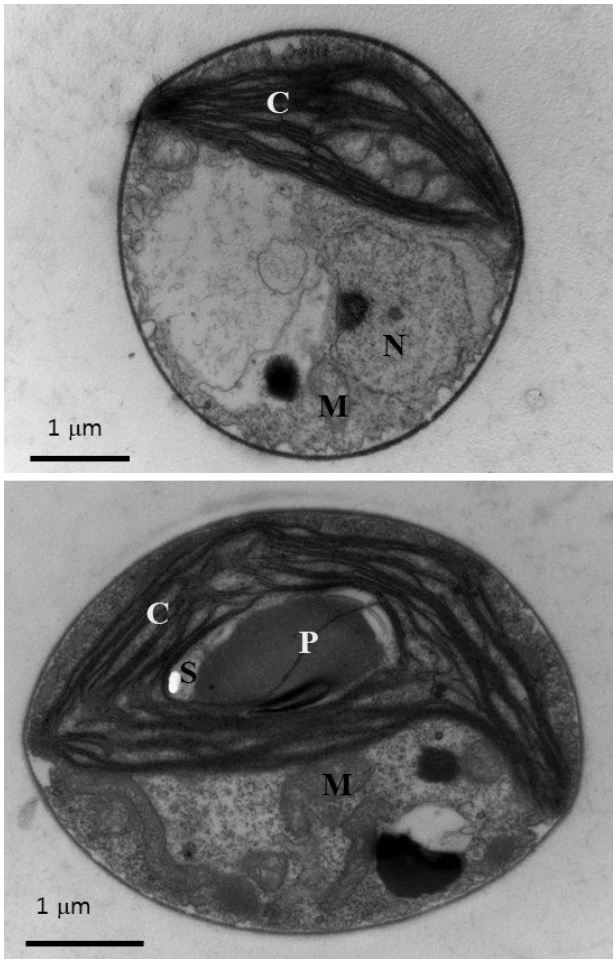


**Fig. 3.** TEM ultrastructure images of *M. singularis* MM0003. C: chloroplast; M: mitochondrion, N: nucleus; P: pyrenoid; S: starch.

## 2. Phylogenetic position determined by genetic markers

Molecular identification results were summarized in Table 2. The 18S rRNA sequence of the isolate was identical to *Micractinium* sp. KNUA034 (KM243325), but the ITS sequence of strain MM0003 was only 97.65% homologous to that of *Micractinium* sp. KNUA034 (KM243327). As illustrated in Fig. 4, strain MM0003 was clustered with other 3 Antarctic *Micractinium* species such as *M. singularis* KSF0094 (MN414469), *M. variabile* KSF0085 (MN 414468), and *Micractinium* sp. KNUA034 (KM243325 +KM243327). The ITS2 secondary structure of 3 *Micractinium* species exhibited typical four helices and pyrimidine-pyrimidine bulge on helix II, the conserved sequence TGGT (UGGU) on the 5′ side of helix III (Mai and Coleman 1997), and a C-G pairing on the top of helix III (Fig. 5). Overall secondary structures of *M. singularis* MM0003 and KSF0094 were very similar to each other and CBC or hemi-CBC was not found in these strains. However, *M. singularis* MM0003 and *M. variabile* KSF 0085 showed differences in helix I structure and these results were in agree with those obtained in the previous publication (Chae *et al.* 2019).

## 3. Optimal growth temperatures and NaCl tolerance for the isolate

As shown in Table 3, the isolate could survive and grow in a temperature range of 5−25°C whilst their optimal growth temperature was 15−25°C. It could not survive over 30°C and delayed growth was also observed at 5°C. Also, the isolate was able to withstand up to 0.5 M NaCl even though growth was suppressed in the presence of NaCl. It did not survive at a concentration over 1.0 M NaCl.

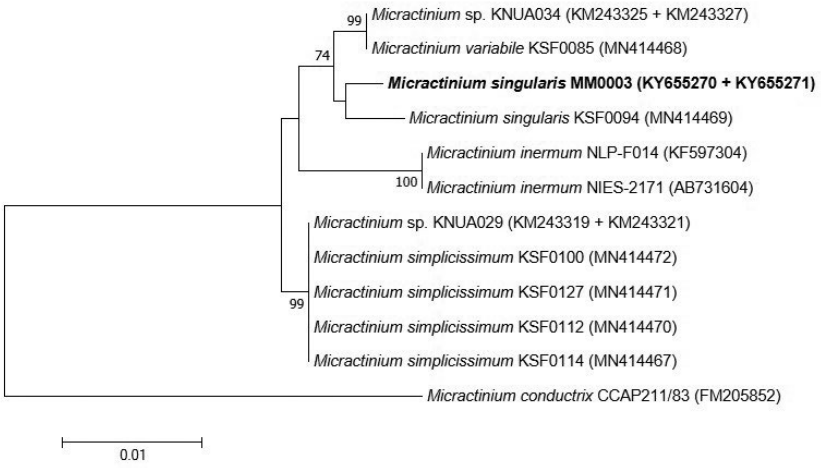


**Fig. 4.** The phylogenetic relationship between strain MM0003 and its closely related species based on the concatenated SSU 18S rRNA and ITS sequences using the K2+G model with *M. conductrix* as an outgroup. The tree was generated by the ML method using 1,000 bootstrap replicates. The scale bar represents a 1% difference in the nucleotide sequences.

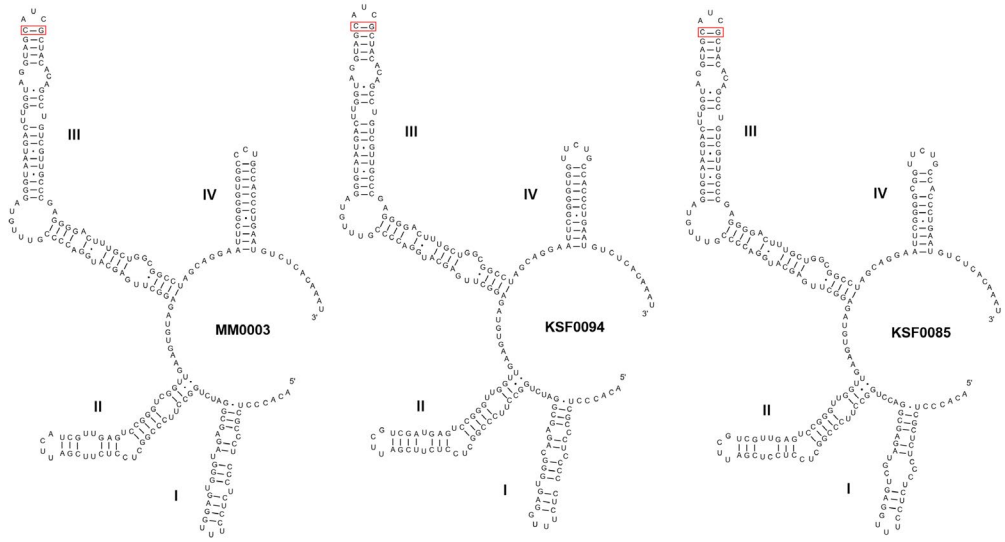


**Fig. 5.** ITS2 secondary structure for *M. singularis* MM0003, *M. singularis* KSF0094, and *M. variabile* KSF0085. The key CBCs at the tip of helix III are indicated in a red box.

**Table 3.** Growth of strain MM0003 at various temperatures

| Temperature (°C) | 5 | 10 | 15 | 20 | 25 | 30 | 35 |
|---|---|---|---|---|---|---|---|
| Growth[a] | + | ++ | +++ | +++ | +++ | − | − |

[a] +++: good growth; ++: moderate growth; +: poor growth; −: no growth.

## 4. Fatty acid composition

The major fatty acids of strain MM0003 were $C_{16:0}$ (26.1 ± 0.2%), $C_{16:4}$ ω3 (12.6 ± 0.3%), and $C_{18:3}$ ω3 (30.9 ± 0.3%). Summarized characteristics for the strain are given in Table 4.

 

## 5. Biomass properties

In proximate analysis by TGA, the moisture content (MC) is determined by the mass loss before 110°C under $N_2$ atmosphere, the organic matter (OM) refers to the mass loss between 110–900°C under $N_2$ as a result of thermal decomposition, and the remaining mass represents the inorganic matter (IM). The TGA profile is shown in Fig. 6 and the material composition of strain MM0003 is presented in Table 6. The GCV and protein content based on the ultimate analysis were 19.8 MJ $kg^{-1}$ and 38.2%, respectively (Table 5).

**Table 4.** Lipid profile of strain MM0003

| Component | Content (%)[a] | Note |
|---|---|---|
| 8-Heptadecene ($C_{17}H_{34}$) | 0.4±0.0 | – |
| Neophytadiene ($C_{20}H_{38}$) | 0.6±0.0 | – |
| Palmitic acid ($C_{16:0}$) | 21.6±0.2 | SFA (major) |
| Palmitoleic acid ($C_{16:1}$ ω7) | 2.8±0.1 | – |
| Hexadecadienoic acid ($C_{16:2}$ ω6) | 1.2±0.1 | – |
| Hexadecatrienoic acid ($C_{16:3}$ ω3) | 2.4±0.0 | |
| Hexadecatetraenoic acid ($C_{16:4}$ ω3) | 12.6±0.3 | Omega-3 PUFA (major) |
| Stearic acid ($C_{18:0}$) | 1.0±0.0 | – |
| Oleic acid ($C_{18:1}$ ω9) | 2.6±0.1 | – |
| Linoleic acid ($C_{18:2}$ ω6) | 7.6±0.3 | – |
| α–Linolenic acid ($C_{18:3}$ ω3) | 30.9±0.3 | Omega-3 PFUA (major) |
| Eicosatetraenoic acid ($C_{20:4}$) | 0.8±0.0 | – |

[a]Values represent the average±standard deviation of three independent experiments.

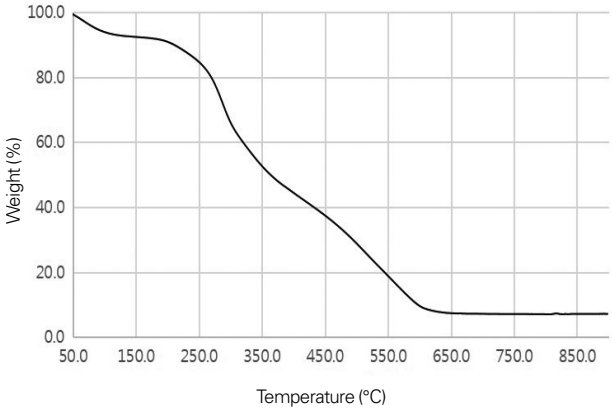


**Fig. 6.** TGA profile of *M. singularis* MM0003.

**Table 5.** Proximate and ultimate analysis results of *M. singularis* MM0003

| Material component | Proximate analysis (wt%) | Elemental composition | Ultimate analysis (wt%) |
|---|---|---|---|
| MC | 6.6 | C | 44.4 |
| OM | 86.1 | H | 6.6 |
| IM | 7.4 | N | 6.1 |
| | | O | 34.8 |
| | | S | 0.8 |
| | | Protein | 38.2 |
| | | GCV (MJ $kg^{-1}$)[a] | 19.8±0.2 |

[a]Value represents the average±standard deviation of two independent experiments.

**Table 6.** Pigment profile of strain MM0003

| Peak number | Retention time | Compound | Content (%)[a] |
|---|---|---|---|
| 1 | 18.2 | Unknown | 6.5±0.1 |
| 2 | 19.8 | Unknown | 4.3±0.0 |
| 3 | 25.3 | Lutein | 30.8±0.0 |
| 5 | 28.8 | Chl-*b* | 13.4±0.1 |
| 6 | 32.0 | Chl-*a* | 38.2±0.1 |
| 7 | 40.9 | β-carotene | 6.8±0.0 |

[a]Values represent the average±standard deviation of three independent experiments.

**Table 7.** Monosaccharide composition of strain MM0003

| Compound | Content | |
|---|---|---|
| | %[a] | mg $g^{-1}$ DW |
| Glucose | 88.8±3.6 | 14.8 |
| Mannose | 9.9±0.7 | 2.9 |
| Mannitol | 1.2±0.1 | 0.6 |

[a]Values represent the average±standard deviation of three independent experiments.

### 6. Pigment composition

The pigment profile of *M. singularis* MM0003 is reported in Table 6. The major pigments of the isolate were Chl-*a* (38.2%±0.1%), lutein (30.8%±0.0%), Chl-*b* (13.4%±0.1%) and β-carotene (6.8±0.0%). The other minor peaks were not identified.

### 7. Monosaccharide composition

The carbohydrate composition of the isolate is summarized in Table 7. Its major monosaccharides were glucose (88.8%, 14.8 mg $g^{-1}$ DW) and a small portion of minor sugars (mannose 9.9% and mannitol 1.2%) were also detected.

### 8. Deposition of the isolate

Stain MM0003 obtained in this study was deposited in the National Marine Biodiversity Institute of Korea (MABIK) and the Korean Collection for Type Cultures (KCTC) under the accession numbers of MABIK-LP-00000134 and KCTC 13290BP, respectively.

## DISCUSSION

In this study, a psychrotolerant Korean *M. singularis* strain was axenically isolated and its identity was analyzed by morphological, molecular, and physiological approaches. Establishment of a pure culture was achieved by the combination of imipenem treatment and physical separation technique. As the isolate only had very unsophisticated cell structures such as the chloroplast, pyrenoid, nucleus, and mitochondrion, it was unable to distinguish strain MM0003 from *Chlorella* spp. by traditional morphological criteria.

However, it was able to determine the phylogenetic position of the isolate by the sequence analyses of SSU and ITS regions. As shown in Fig. 4, the isolate showed close relationship with the newly described Antarctic *Micractinium* species (Chae *et al.* 2019). In addition, the key CBCs in the ITS2 secondary structure also confirmed that strain MM0003 belonged to the genus *Micractinium* (Hoshina *et al.* 2010). Strain MM0003 exhibited typical four-fingered hands (helices I through IV) and a C-G pairing on the top of helix III (Fig. 5). The predicted ITS2 secondary structure diagrams for the closely related *Micractinium* species also confirmed that strain MM0003 possessed similar secondary structure to *M. singularis* KSF0094 isolated from Deception Island, South Shetland in Antarctica (Chae *et al.* 2019). Furthermore, strain MM0003 exhibited the closest affinity with the non-bristle-forming *M. singularis* KSF0094. Since both Antarctic *Micractinium* strains did not produce bristle under grazing pressure and possess solitary habit, it can be concluded that they share similar morphological, physiological, and molecular characteristics with each other. Hence, strain MM0003 was identified as *M. singularis* and this is the first report of this species in Korea.

The isolate was capable of surviving and propagating as low as at 5°C showing its cryotolerant properties. However, the optimum growth temperature was 15−25°C which indicates that this Korean *M. singularis* strain is mesophilic. It has been reported that the maintenance of membrane fluidity by the unsaturation of the fatty acids in membrane lipids under cold temperatures is one of the main adaptation strategies of polar microalgae (Osipova *et al.* 2009; Boelen *et al.* 2013). Analysis of the cellular fatty acid composition of strain MM0003 also revealed that it was rich in $C_{16:0}$ (21.6%) saturated fatty acid (SFA) and $C_{18:3}$ ω3 (30.9%) and $C_{16:4}$ ω3 (30.6%) polyunsaturated fatty acids (PUFAs). Beneficial health effects of essential omega-3 PUFAs were well documented by Mehta *et al.* (2009). Currently omega-3 PUFAs are derived from mainly marine biological resources, such as fish oil, and various commercial products are available worldwide. Given the current ocean pollution and overfishing issues, as well as global climate change, the sustainability of marine fish as a safe resource of omega-3 PUFAs for human consumption is in great doubt (Kang 2011; Jeromson *et al.* 2015). Therefore, this marine microalga may serve as potential biological resource that can replace fish-based oil for sustainable and pollution-free omega-3 PUFAs production.

The OM can be defined as the part of solid fuel that is driven-off as a gas by heating and typical biomass gener-

 

ally has a OM content of up to 80% (crop residue: 63–80%; wood: 72–78%). The OM content of this microalga (86.1%) used in this study was higher than the ranges of plant- and wood-based biomass feedstocks. The GCV was also calculated to understand the potential of algal biomass as a biofuel feedstock (Table 5). The results showed that the GCV was within the range of the terrestrial energy crops (17.0 – 20.0 MJ $kg^{-1}$) (Ross *et al*. 2008). Since fine particulate matters have become a national concern in recent years, some of coal-burning power stations in Korea have converted to biomass-burning stations and many plants are considering this move in the near future. Hence, microalgae pellet made of mass-cultivated microalgae biomass would be an excellent mixed combustion biofuel for these coal power stations.

Pigment analysis results indicated that strain MM0003 was able to autotrophically biosynthesize Chl-*a*, lutein, and Chl-*b* as its major pigments. In particular, lutein is an extensively used antioxidant carotenoid in the food, pharmaceutical, and cosmetics industries and it has recently received increased attention due to the beneficial effects on eye health (Buscemi *et al*. 2018). Currently, commercial sources are mainly extracted from marigold flowers, but the lutein content in *Tagetes erecta* is low and it was also reported that the lutein yields are very low (Ausich 1997; Piccaglia *et al*. 1998; Del Campo *et al*. 2007; Vechpanich and Shotipruk 2011). Therefore, mass-cultivated biomass of *M. singularis* MM0003 under controlled conditions could serve as an alternative source of lutein.

It was reported that quantitative and qualitative analyses of simple sugars in microalgae are essential steps for the optimal utilization of microalgal biomass (Ortiz-Tena *et al*. 2016). Carbohydrate analysis revealed that the major monosaccharide of the isolate was glucose ($88.8 \pm 3.6\%$) and mannose ($9.9 \pm 0.7\%$) and mannitol ($1.2 \pm 0.1\%$) were also identified as minor components. Since glucose is a preferred carbon source for *Saccharomyces cerevisiae*, residual biomass of strain MM0003 after extraction of valuable components from microalgae could be hydrolyzed into fermentable sugars for bioethanol production via fermentation. This finding would also contribute to a better understanding of the diversity of microalgal monosaccharides.

The physiological characteristics reported in this study offer in-depth insight into chemotaxonomic markers, as well as morphological and molecular characteristics, of the isolated *M. singularis* strain. Moreover, the results may serve as foundation for the future improvements in the production of high-value products.

In this study, we provided the first record of *M. singularis* in Korea on the basis of the morphological, molecular, and physiological data. It can be concluded that this marine microalga may serve as a potential biological resource for producing biochemicals of commercial interest as well as a promising candidate for further phylogenetic and evolutionary studies in both industrial and academic fields. Also, there are still a large number of domestic Trebouxiophyceae remained unknown (Kim *et al*. 2018), further efforts are required to explore the diversity of Trebouxiophyceae in Korea.

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