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First record of a marine microalgal species, *Jaagichlorella roystonensis* (Trebouxiophyceae) isolated from Jungmun Saekdal Beach, Jeju Island, Korea

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Abstract: A eukaryotic marine microalga was isolated from Jungmun Saekdal Beach, Jeju Island, Korea and an integrated approach, including molecular phylogeny and morphology, was used to determine its taxonomical status. Molecular phylogenetic evidence inferred from the small subunit (SSU) 18S rRNA sequence and internal transcribed spacer (ITS) secondary structure analysis clearly showed that the isolate belonged to the recently described species, *Jaagichlorella roystonensis*. Distinctive morphological keys of the species were also observed by light microscopy and scanning/transmission electron microscopy (S/TEM). In this study, a Korean marine *J. roystonensis* species was described for the first time and was subsequently added to the national culture collections in Korea.

Keywords: first record, *Jaagichlorella roystonensis*, Jungmun Saekdal Beach, Korean marine microalga

INTRODUCTION

Jaagichlorella was first established and described by Reisigl (1964) and the genus was morphologically characterized by its ellipsoidal cells with a plate-shaped chloroplast with a single pyrenoid, but there has not been much addition to the members of this genus until recently. However, new species have recently been isolated and described or transferred from other taxonomic groups with an aid of molecular phylogeny. In particular, the genus was reestablished as *Jaagichlorella* for *Heveochlorella* based on the secondary structures of the marker genes (Darienko and Pröschold 2019). Currently, there are 6 taxonomically accepted species in this genus including *J. africanam*, *J. geometrica* (type species), *J. hainangensis*, *J. luteoviridis*, *J. roystonensis*, and *J. sphaerica* (Guiry and Guiry 2020).

In this study, a unicellular microalga belonging to the genus *Jaagichlorella* was isolated from Jungmun Saekdal Beach in Korea and an axenic culture was established. Its phylogenetic position based on the SSU rRNA sequence analysis and ITS2 secondary structure prediction showed that the isolate was clustered with the recently proposed *J. roystonensis* strains (Darienko and Pröschold 2019). In conclusion of our findings, we report information on the first record of this species in Korea and its morphological and molecular characteristics.

MATERIALS AND METHODS

1. Sample collection and isolation of microalga

Seawater samples were collected from Jungmun Saekdal Beach in Saekdal-dong, Seogwipo, Jeju, Korea in September of 2017. The location and physico-chemical data of the sampling sites 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 BG-11 agar plates (UTEX, Austin, TX, USA) supplemented with 100 μg mL^{-1} imipenem (Sigma-Aldrich, St. Louis, MO, USA) to suppress contaminating bacterial growth and generate axenic cultures (Kang *et al.* 2019). The plates were then incubated at 20°C in a growth chamber (FLI-2010A, Eyela, Tokyo, Japan) with cool fluorescent light (approximately 30 μmole m^{-2} 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 a pure culture was produced. Once an axenic culture was established, a single colony was transferred onto a fresh R2A agar plate (Becton, Dickinson and Company, Sparks, MD, USA) also supplemented with imipenem (20 μg mL^{-1}).

2. Morphological identification

A single colony was transferred in 100 mL R2A medium in a 250-mL Erlenmeyer flask and the flasks were incubated at 20°C. Well-grown live cells were observed by an upright microscope (Microscope Axio Imager.A2, Carl Zeiss, Göttingen, Germany).

For SEM, 10 mL aliquots of cultures at around 1,000 cells mL^{-1} were fixed for 10 min in osmium tetroxide (OsO_4, Electron Microscopy Sciences, EMS hereafter, 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 an automated critical point dryer (EM CPD300; Leica, Wetzlar, Germany). The dried filters were mounted on an aluminum stub (EMS) using copper conductive doubled-side tape (Ted Pella, Redding, CA, USA) and coated with gold in an ion sputter (MC1000; Hitachi, Tokyo, Japan). 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 1.5 hrs and the content was concentrated at 1,610 g for 10 min in a Vision Centrifuge VS-5500 (Vision Scientific, Bucheon, Korea). The resulting pellet was subsequently transferred to a 1.5 mL tube and rinsed in 0.2 M sodium cacodylate buffer (EMS) at pH 7.4. After several rinses in 0.2 M sodium cacodylate 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, Ansan, Korea). 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 (EMS). Sections were prepared on an EM UC7 ultramicrotome (Leica) and stained with 3% (w/v) aqueous uranyl acetate (EMS) followed by lead citrate (EMS). 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) and further purified by a Wizard DNA Clean-Up System (Madison, WI, USA) to get rid of possible polymerase chain reaction (PCR) inhibitors. 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 used in this study and the DNA sequencing were carried out at the Macrogen facility (Daejeon, Korea). Phylogenetic analysis was performed with the 18S rRNA sequences using the software package Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 (Kumar *et al.* 2016). The 18S rRNA sequence of the isolate was aligned with those of the 13 close rela-

Table 1. Description of the sampling site

Depth (m)	Temperature (°C)	Salinity (PSU[a])	Latitude	Longitude
0.3	17.0	32.8	33°14′37.91″N	126°24′50.90″E

[a]PSU: practical salinity unit

tive strains based on the previous publication (Darienko and Pröschold 2019) using ClustalW incorporated in MEGA 7.0. package. Its 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 + evolutionarily invariable, K2 + G + I) 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. Three *Kalinella* strains were used as an outgroup. DNA sequences obtained in this study were deposited in the NCBI under accession numbers MN960106 and MK182291 (Table 2). The ITS2 secondary structures were constructed using Mfold (Zuker 2003) according to Darienko *et al.* (2016).

4. 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. 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 estimated from the N content in the ultimate analysis by using the conversion factor of ×6.25 (Mariotti *et al.* 2008) and C/N ratio was also calculated by dividing total carbon percentage with total nitrogen percentage.

Table 2. BLAST search results (searched date: 08 March 2020) using the 18S rRNA and ITS sequences of *J. roystonensis* MM0044

Marker gene	Accession No.	Length (bp)	Closest match (GenBank accession No.)	Overlap (%)	Sequence similarity (%)
18S rRNA	MN960106	1,767	*Jaagichlorella roystonensis* SAG 2133 (MH780940)	100	99.55
ITS	MK182291	737	*Jaagichlorella roystonensis* ITBB A3-8 (JX290371)	98	99.72

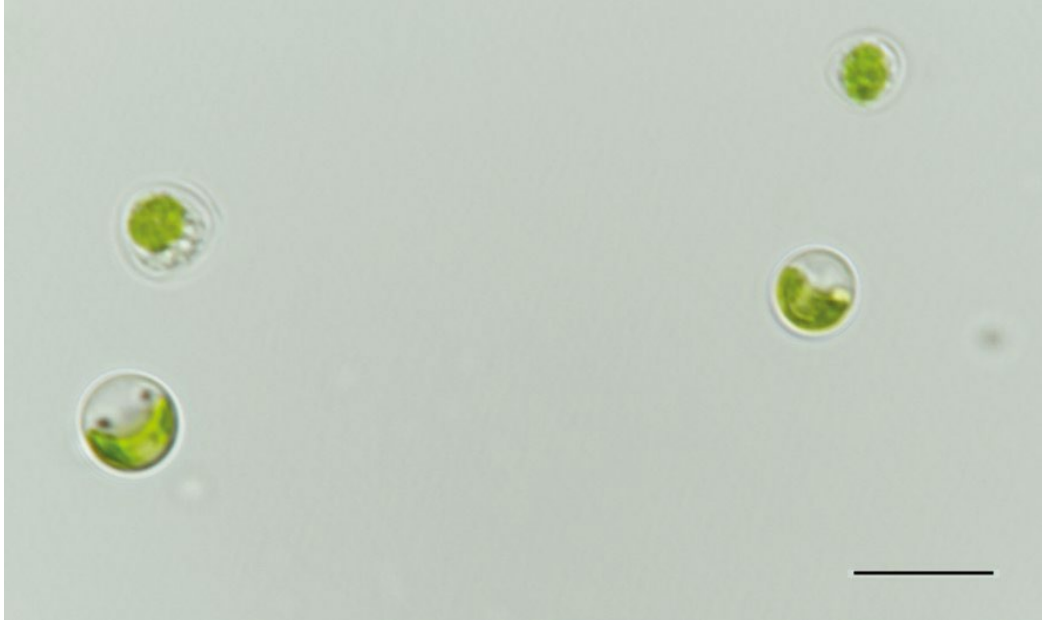

Fig. 1. Light microscopy of *Jaagichlorella roystonensis* MM0044. Scale bar = 10 μm.

RESULTS

1. Morphology of the isolate

As shown in Fig. 1 and Fig. 2, the microalgal cells were solitary and round to slightly ellipsoid in shape and their sizes ranged from approximately 4 to 8 μm in diameter. Cytological observation showed that the cells had a parietal cup-shaped chloroplast containing one pyrenoid located in the center of the chloroplast (Fig. 3). The pyrenoid was penetrated by radially arranged tubular invaginations and the nucleus and mitochondrion were also observed in the cells (Fig. 3). Also, the cells had smooth and two-layered walls and no mucilage were found.

2. Phylogenetic position determined by genetic markers

Molecular identification results were shown in Table 2. The 18S rRNA and ITS sequences the isolate were 99.55% homologous to that of *J. roystonensis* SAG 2133 (MH780940) and 99.72% homologous to that of *J. roystonensis* ITBB A3-8 (JX290371), respectively. As illustrated in Fig. 4, strain MM0044 was clustered with other *J. roystonensis* strains such as SAG 2196 (MH780941), SAG 2133 (MH780940), SAG 2198 (MH780942), and ITBB

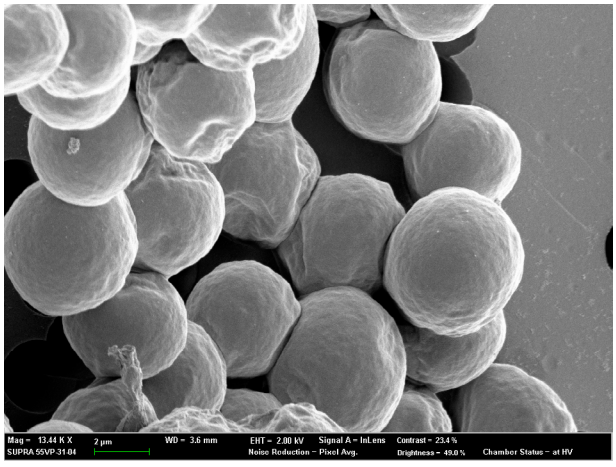

Fig. 2. FE-SEM image of *Jaagichlorella roystonensis* MM0044.

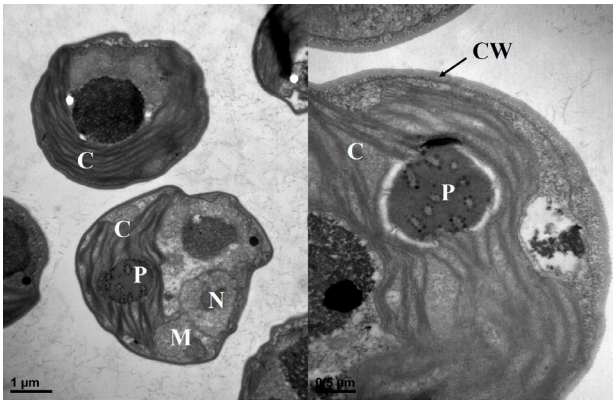

Fig. 3. TEM micrographs of *Jaagichlorella singularis* MM0044; C: chloroplast; CW: cell wall; M: mitochondrion, N: nucleus; P: pyrenoid.

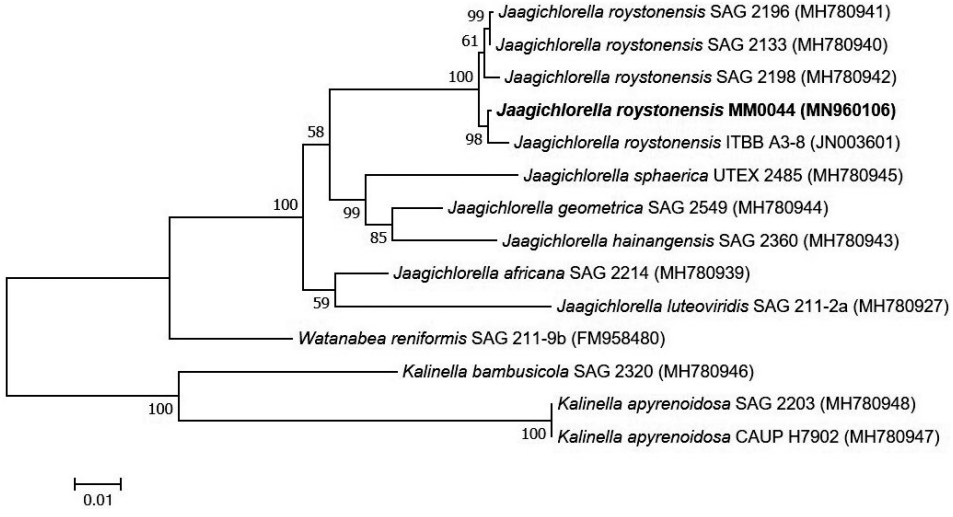

Fig. 4. The phylogenetic relationship between strain MM0044 and its closely related species based on the SSU 18S rRNA using the K2 + G + I model with *Kalinella* spp. as an outgroup. The tree was generated by the maximum-likelihood (ML) method using 1,000 bootstrap replicates. The scale bar represents a 1% difference in the nucleotide sequences.

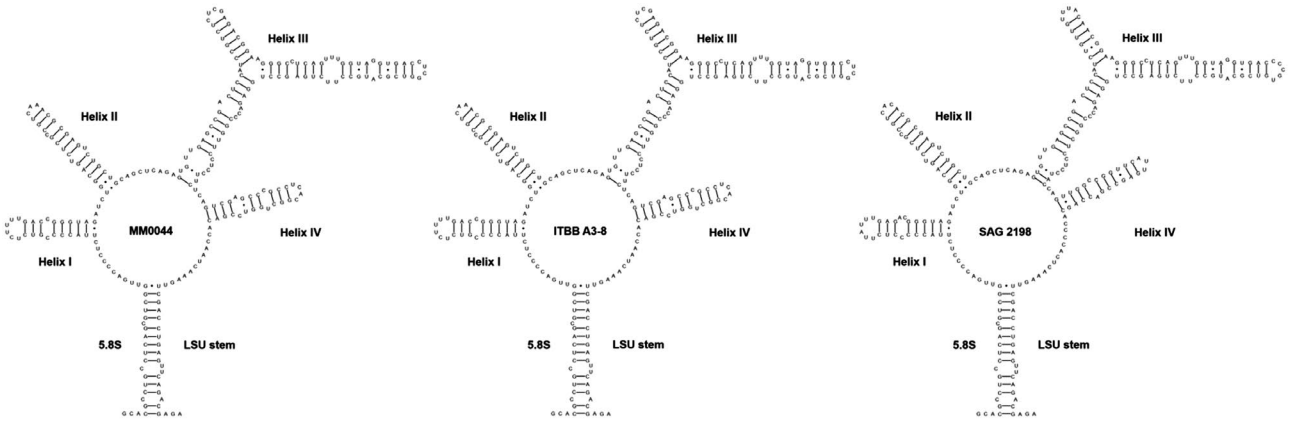

Fig. 5. ITS2 secondary structures for *Jaagichlorella roystonensis* MM0044, *J. roystonensis* ITBB A3-8, and *J. roystonensis* SAG 2198.

Table 3. Ultimate analysis results of *J. roystonensis* MM0044

Elemental composition	Ultimate analysis (wt%)[a]
C	46.1±0.1
H	7.1±0.0
N	9.1±0.1
S	0.6±0.0

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

A3-8 (JN003601). The ITS2 secondary structures of three *J. roystonensis* strains were investigated and the structures of *J. roystonensis* MM0003 and ITBB A3-8 (JX290371) were identical to each other (Fig. 5).

3. Biomass properties

The elemental composition of strain MM0044 was presented in Table 5. In addition, the GCV, protein content, and C/N ratio based on the ultimate analysis were 19.4 MJ kg^{-1}, 56.6%, and 5.1, respectively.

4. Deposition of the isolate

Stain MM0044 obtained in this study was deposited in the National Marine Biodiversity Institute of Korea (MABIK) and in the Korean Collection for Type Cultures (KCTC) under the accession numbers of MABIK-LP-00000103 and KCTC 13748BP, respectively.

DISCUSSION

In this study, a pure culture of a Korean *J. roystonensis* strain was established and its identity was mainly analyzed by morphological and molecular approaches. According to the original description by Reisigl (1964), the only difference between *Jaagichlorella* and *Chlorella* is the type of chloroplast: *Jaagichlorella* had a plate-shaped chloroplast while *Chlorella* had a cup-shaped one. Even though this kind of approach is no longer valid for species delimitation, the oldest generic genus name, *Jaagichlorella*, has priority over younger synonyms according to the International Code for Nomenclature (ICN).

Since the emergence of the *Watanabea* clade in Trebouxiophyceae (Karsten *et al.* 2005) with an aid of the molecular taxonomy approaches, several new genera including *Heveochlorella* have been classified, discovered, or revised as members of this clade (Zhang *et al.* 2008; Darienko and Pröschold 2019). Ma *et al.* (2013) isolated the second species of *Heveochlorella* from the bark of the royal palm tree (*Roystonea regia*) and they described is as *H. roystonensis*. The genus was then reestablished as *Jaagichlorella* according to the phylogenic analyses based on the secondary structures of the marker genes (Darienko and Pröschold 2019). Likewise, 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 relationships with the newly revised *J. roystonensis* strains (Darienko and Pröschold 2019). In addition, the ITS2 secondary structure prediction results also confirmed that strain MM0044 belonged to *J. roystonensis* (Fig. 5).

The microalgal cells exhibited similar morphological criteria such as chloroplast, pyrenoid, cell walls etc. with *J. roystonensis*. Hence, strain MM044 was identified as *J. roystonensis* and this is the first report of this species in Korea. All the previously reported *J. roystonensis* strains originated

from China, Germany, and Japan are known as terrestrial species (Darienko and Pröschold 2019; Guiry and Guiry 2020). However, strain MM0044 is the first strain belonging to this species derived from marine aquatic environment of Korea.

The GCV was also calculated to understand the potential of microalgal biomass as a biofuel feedstock and the GCV of the isolate was within the range of the terrestrial energy crops (17.0–20.0 MJ kg^{-1}) (Ross *et al.* 2008). Due to the fine particulate matter concerns in Korea, some of the old Korean coal-burning power stations have been modified to the biomass-burning stations and many other plants nearing the end of their lives are also considering this kind of conversion 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.

In this study, we provided the first record of *J. roystonensis* in Korea on the basis of the morphological and molecular data. It could also be noted that this marine microalga may serve as a potential biological resource for producing biofuel as well as a promising candidate for further phylogenetic and evolutionary studies in the related fields. There are still a large number of domestic Trebouxiophyceae remained undiscovered (Kim *et al.* 2018), further research is required to explore the diversity of Trebouxiophyceae in Korea.

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