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The Discovery and Characterization of a Novel Microalgal Strain, *Picochlorum* sp. KCTC AG61293, with Potential for α-Linolenic Acid Production

Sungmo Kang [1,2], Hyeon Ho Shin [3,*] and Zhun Li [1,4,*]

[1] Biological Resource Center/Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, Jeongeup 56212, Republic of Korea; ksm@kribb.re.kr
[2] Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea
[3] Library of Marine Samples, Korea Institute of Ocean Science & Technology, Geoje 53201, Republic of Korea
[4] Department of Environmental Biotechnology, University of Science and Technology (UST), 217 Gajeong-ro, Yuseong, Daejeon 34113, Republic of Korea
* Correspondence: shh961121@kiost.ac.kr (H.H.S.); lizhun@kribb.re.kr (Z.L.); Tel.: +82-55-639-8440 (H.H.S.); +82-63-570-5628 (Z.L.)

Abstract: Microalgae are highly valued for their rapid biomass production and metabolite synthesis, as well as their abundance of beneficial compounds. They have a variety of applications, including serving as the primary ingredient in biofuels, functional foods, and cosmetics. The genus *Picochlorum*, which was established to represent the unique characteristics of "*Nannochloris*-like" algae, exhibits rapid growth and a high salt tolerance. The morphology, molecular phylogeny, and fatty acid composition of an unspecified *Picochlorum* strain KCTC AG61293 found in Korean coastal waters were investigated. The strain exhibited a unique cell morphology and reproduction type compared to other *Picochlorum* species, as determined using light microscopy, fluorescence microscopy, and field emission scanning electron microscopy (FE-SEM). The vegetative cells were elongated and cylindrical in shape, underwent binary fission, and possessed a parietal chloroplast. A molecular phylogenetic analysis using nuclear small subunit ribosomal RNA sequences showed that *Picochlorum* sp. (KCTC AG61293) belongs to the *Picochlorum* clade and is closely related to the genus *Nannochloris*. Compared to other reference species, *Picochlorum* sp. (KCTC AG61293) had higher levels of saturated fatty acids (SFAs) and alpha-linolenic acid (ALA). The increased levels of SFAs and ALA suggest that *Picochlorum* sp. (KCTC AG61293) may be a promising candidate for biofuel production and other industrial uses.

Keywords: KCTC AG61293; fatty acids; microalgae; phylogenetic analysis

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1. Introduction

Microalgae are a widely distributed group of single-cell eukaryotic plants that have the potential to serve as a source of biomass and raw materials [1,2]. They offer a variety of beneficial substances, including fatty acids (FAs) [3], chlorophylls [4], and carotenoids [5], making them highly valuable in numerous industries. The rapid production of useful metabolites and biomass by microalgae has been widely recognized. Microalgae offer several advantages, including rapid growth rates, efficient land utilization, carbon dioxide (CO_2) sequestration, and the ability to be cultivated in wastewater [6]. Despite their microscopic size, microalgae can produce significant amounts of lipids using simple and rapid cultivation methods [7,8]. These lipids, a key component of microalgae, serve as the primary ingredient in biofuels, functional foods, and cosmetics [9–12]. For instance, palmitic acid (C16:0) is a type of saturated fatty acid that plays a significant role in the production of biodiesel [13]. α-Linolenic Acid (ALA) is a crucial omega-3 fatty acid that is vital for human growth and development [14]. ALA, which is mainly found in plant-based foods, is an essential precursor to eicosapentaenoic acid (EPA) or docosahexaenoic acid

(DHA) [15]. Microalgae, such as *Picochlorum* and *Nannochloropsis*, are particularly significant due to their euryhaline nature and high eicosapentaenoic acid (EPA) content [16,17]. These unique characteristics make them viable candidates for applications in the nutraceutical and biodiesel production sectors [18,19].

Picochlorum species, in particular, have been recognized for their potential in biodiesel production due to their fast growth rates and high lipid contents. *Picochlorum* species are considered as promising candidates for renewable fuel production [20]. A significant study identified Picochlorum as a prime candidate for biodiesel due to its ability to produce substantial lipid quantities, a key component of biodiesel [21]. In other research, such as a study on the BDUG 100241 strain of *Picochlorum* sp., the focus has been on optimizing cultivation conditions to increase biomass productivity and thereby increase biodiesel yield [22].

Similarly, other research, such as that on *Acutodesmus dimorphus*, has explored the growth of specific microalgae in specialized media to maximize biomass yield and contribute to potential biodiesel production [23]. In addition, other studies have highlighted innovations in microalgal cultivation, such as the use of naturally floating microalgal mats, which provide an efficient approach to biomass production for biofuel [24].

The genus *Picochlorum* was established to accurately reflect the phylogenetic relationships and unique characteristics of '*Nannochloris*-like' algae [25]. It was created to accommodate certain marine or saline autosporic taxa that were previously classified as either *Nannochloris* or *Nanochlorum*. These taxa, along with several others, were found to belong to a diverse sister clade to a clade that included the four "true" *Chlorella* species [25]. The decision to create the new genus *Picochlorum* was based on its distinct freshwater and marine or saline lineages, which comprise at least three major subclades, generally corresponding to cell division patterns [25–27]. *Picochlorum* strains exhibit a faster exponential growth rate compared to other commonly used microalgae such as *Dunaliella* and *Nannochloropsis* [28,29]. They have a high salt tolerance [30,31] and can survive in temperatures ranging from 0 to 40 °C [32,33]. These strains can also accumulate lipids, ranging from 20 to 58% of their dry weight [34,35].

Although there is clear evidence of convergent evolution in '*Nannochloris*-like' green microalgae, their identification using microscopy techniques, such as transmission electron microscopy (TEM) or scanning electron microscopy (SEM), remains challenging. Therefore, a widely accepted approach for a more natural classification is to integrate morphological, ecophysiological, and molecular phylogenetic methods [36].

During our scientific expedition along the Korean coast, we discovered a new strain of *Picochlorum*, which we named *Picochlorum* sp. (KCTC AG61293). This discovery expands the variety of *Picochlorum* strains and shows great potential for industry. We used state-of-the-art techniques for our research, including light and SEM for morphological studies and nuclear small subunit ribosomal RNA sequences for phylogenetic studies. The isolate displayed distinct, unique morphological characteristics that differed from known cell division patterns in the genus *Picochlorum*. Both morphological and molecular phylogenetic analyses confirmed that the genus *Picochlorum* is not limited to autosporic taxa. We assessed the strain's ability to produce fatty acids and compared it with that of other strains. In particular, *Picochlorum* sp. (KCTC AG61293) exhibited significantly higher concentrations of omega-3 fatty acids, specifically ALA, compared to other *Picochlorum* genera. This highlights its potential as an environmentally friendly source of lipid products, making it a strong candidate for various industrial applications. Our study significantly advances the characterization of '*Nannochloris*-like' green microalgae and presents opportunities for future research and industry exploitation.

2. Materials and Methods

2.1. Sampling and Cultivation

On 24 May 2023, a sample of chlorophyte was collected from the Marado coast of South Korea (33°07′02″ N, 126°16′05″ E) using a plankton net with a mesh size of 20 µm.

The temperature and salinity at the time of collection were 24 °C and 30‰, respectively. Cells that appeared similar to *Picochlorum* were isolated using a capillary pipette (3151102, Hilgenberg, Malsfeld, Germany) under a light microscope (Axio Imager A2, Carl Zeiss, Oberkochen, Germany) after multiple rinses with sterile seawater droplets [37,38]. These cells were then placed in a tissue culture plate with 48 wells (32048, SPL Life Sciences, Pocheon, Republic of Korea) filled containing the culture medium (f/2; G0154, Sigma Aldrich Co., Ltd., St. Louis, MO, USA). Later, the cells were transferred to a cell culture flask (70025, SPL Life Sciences, Pocheon, Republic of Korea) and a 24-well tissue culture plate (32024, SPL Life Sciences, Pocheon, Republic of Korea) using a micropipette (ACURA 826, Socorex Isba SA, Ecubleus, Switzerland) and a capillary pipette. The cells were cultured under cool white illumination at an intensity of 50 μE/m/s and a temperature of 23 °C with a light:dark cycle of 12:12 h. The analyzed cells were in the exponential growth phase and had been cultured for one week. They were deposited at the Korea Collection for Type Cultures (KCTC) of the Korea Research Institute of Bioscience & Biotechnology and the National Marine Biodiversity Institute of Korea (MABIK).

2.2. Light Microscopy

Live cells of *Picochlorum* sp. (KCTC AG61293) were examined and images were captured at 1000× magnification using an Axiocam Color 512 camera (Carl Zeiss, Oberkochen, Germany) connected to an Axio Imager A2 microscope (Carl Zeiss, Oberkochen, Germany). Using a microscope application program, the dimensions of 300 individuals were measured using the GEN 3.3 program (Carl Zeiss, Oberkochen, Germany). The living cell culture was pre-suspended in a 2.5% glutaraldehyde fixative solution (Electron Microscopy Sciences, Hatfield, PA, USA) in a microcentrifuge tube (EMT-1530, Biofact, Daejeon, Republic of Korea) for fluorescence microscopy. A drop of NucBlue™ Fixed Cell ReadyProbes™ DAPI reagent (R37606, Molecular Probes, Eugene, OR, USA) was added and the cells were dyed for the detection of internal lipids with Nile Red reagent (72485, Sigma Aldrich Co., Ltd., St. Louis, MO, USA) dissolved in DMSO solution (472301, Sigma Aldrich Co., Ltd., St. Louis, MO, USA). The samples were then kept in the dark for 30 min at ambient temperature. Chlorophyll a, DAPI-stained nuclei, and lipids were imaged using an Axiocam Mono 503 camera (Carl Zeiss, Oberkochen, Germany) attached to an Axio Imager microscope (Carl Zeiss, Oberkochen, Germany) with a high-performance LED light engine (KFM-IS3, Korealabtech, Seongnam, Republic of Korea).

2.3. Scanning Electron Microscopy (SEM)

A solution from the mid-growth-phase batch culture was stabilized at room temperature for 30 min with a fixative solution (OsO_4; 2% final concentration; Sigma Aldrich Co., Ltd., St. Louis, MO, USA) for the SEM analysis. Several rinses with distilled water were then performed on the stabilized cells. Following the rinses, the specimens were dewatered through a gradient set of concentrations of ethanol (100983, Merck KGaA, Darmstadt, Germany) (ranging from 10 to 99% in seven steps), with each step lasting 10 min. The samples were then subjected to critical point drying by using a critical point dryer (Autosamdri®-815, Tousimis, Rockville, MD, USA) with CO_2 in liquid form. Lastly, the samples were platinum-covered with a CCU-010 coater (Safematic GmbH, Zizers, Switzerland) and imaged using a Regulus 8100 field emission SEM (Hitachi, Tokyo, Japan).

2.4. Extract DNA, PCR, and Sequencing

Extracted genomic DNA was prepared from a 1 mL culture medium of *Picochlorum* sp. (KCTC AG61293) in the exponential growth phase using a Tissue Lyser (Qiagen, Hilden, Germany) and a Dneasy® Powersoil kit (Qiagen, Hilden, Germany), according to the guidelines of the kit manufacturer. Amplification of the nuclear SSU (18S rRNA) gene sequences was performed using Green1 and Green2 primers [39]. Additionally, the chloroplast rRNA (16S rRNA) gene sequences were amplified using pA and B23s primers [40]. The PCR was performed on a Mastercycler® nexus (Eppendorf, Hamburg,

Germany) with the use of KOD-ONE™ Master Mix (KMM-101, TOYOBO, Osaka, Japan). The procedure included an initial phase at 94 °C for 2 min, followed by 30 cycles of denaturation for 10 s at 98 °C, 30 s of annealing at 55 °C, 1 min of extension at 68 °C, and 5 min of the last extension phase at 68 °C. The products were verified using gel electrophoresis on 1% agarose, and then purification was performed using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The cycle sequencing reaction was carried out with the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (43-374-55, Applied Biosystems, Foster City, CA, USA).

2.5. Sequence Alignment and Phylogenetic Analysis

The 1642 bp nssu rRNA gene sequence was compared with the sequences of the genus *Picochlorum* and its associated taxa deposited in GenBank (Table S1). These were sequenced using ClustalW with standard settings, and the alignment was imported into MEGA 11 [41], resulting in a final alignment of nssu 1748 bp, including the introduced gaps. A maximum likelihood (ML) analysis and Bayesian inference (BI) were used to construct phylogenetic trees from this sequence data set. RaxML ver. 8 [42] was used for the ML analysis, with a General Time Reversible (GTR) parameter model allowing for γ-distributed rate variation across sites (G). Statistical reliability was assessed using an ML bootstrap analysis with 1000 re-sampling. The Akaike information criterion, which was implemented in jModelTest v2.1.4 [43], was used to select the GTR + I + G substitution model for Bayesian inference. Inference was performed using MrBayes 3.2 [44], which allowed for one invariant site and six classes of gamma rays. Four Markov chain Monte Carlo (MCMC) simulations were run for 10 M generations, with a sample taken every 100 generations. The initial 10,000 trees were burnt in and discarded. A consensus majority-rule tree was then built to examine the posterior probability of each clade. Visualization of the final tree was achieved using the online tool iTOL v6 [45].

2.6. FA Methyl Ester Profile Analysis

During the culture's exponential phase, cells were gathered using centrifugation at 12,000× rpm for 10 min at 25 °C. The cells were then meticulously washed with deionized water to eliminate saline and freeze-dried at −80 °C overnight to obtain dry biomass. A revised method of direct transesterification suggested by Lepage and Roy [46] was used. The dry biomass (under 50 mg each) was mixed with 100 µL of an internal standard solution of methyl heptadecanoate (51633, Sigma Aldrich Co., Ltd., St. Louis, MO, USA) dissolved in n-hexane (270504, Sigma Aldrich Co., Ltd., St. Louis, MO, USA) (IS; con. 3 mg/mL) and 0.9 mL of 5% (*v*/*v*) acid catalyst (acetyl chloride/methanol). A ThermoMixer® C (Eppendorf, Hamburg, Germany) was utilized to let the reaction occur at 80 °C for 1 h. After the reaction, 1 mL of n-hexane was added to the reaction tube to blend and transfer the FAME-reactive substance to the layer of n-hexane. The FAME profile was verified by adding the supernatant to an analysis vial. The methods and conditions for the FAME analysis by GC were taken from our previous study [47]. The contents and compositions of FAME were examined using a gas chromatograph (GC-2010 plus, Shimadzu, Kyoto, Japan) instrumented with a capillary column (thickness: 0.25 µm, diameter: 0.25 mm, and length: 30 m; Agilent J&W DB-23, 122-2361, Santa Clara, CA, USA) and a flame ionization detector (FID). Nitrogen gas was used at a flow rate of 3 mL/min, and a pressure of 96.7 kPa was used as the purge flow. The injected volume was 1 µL and the injector split ratio was 25:1. The split injector and FID conditions were 250 °C and 280 °C, respectively. The running time of analysis was 31 min. The oven condition was initially set to a temperature of 50 °C and maintained for 1 min. It was then heated up to 175 °C at a rate of 25 °C/min. Following this, the temperature was further increased to 205 °C at a slower rate of 2 °C/min. The oven was then kept at 205 °C for a duration of 5 min. Finally, the temperature was slowly increased at a rate of 1 °C /min until it reached the terminal temperature of 210 °C. A qualitative analysis of FAME was performed by comparing the peak retention times of the Supelco 37 component FAME Mix (CRM47885, Merck KGaA, Darmstadt, Germany). The

peaks (below C14:0) were excluded from the peak area measurements obtained by the GC analysis, and the FAME contents were determined from the peak area measurements and the weight of sample (DCW; Dry cell weight) according to the formula (1).

$$FA\ content(\%) = \frac{\left(\frac{FAME\ area - IS\ area}{IS\ area}\right)(IS\ con.)(IS\ dilution\ factor)}{DCW\ in\ sample} \times 100 \quad (1)$$

All experiments were performed in triplicate. The signal data at each retention time were compared with those of the IS for a quantitative analysis. The data were processed and visualized with Microsoft Excel (Microsoft Co., Ltd., Redmond, WA, USA).

3. Results and Discussion

3.1. Morphological Features

The vegetative cells were elongated, cylindrical (Figure 1a–e), and highly variable in size (Figure 1a). The cells ranged from 0.8 to 3 µm in length and from 0.8 to 1.7 µm in width, with a length to width ratio of 1.3–1.6. Elongated cells were divided into two cells via binary fission (Figure 1f–h), and the divided cells were spherical in shape (Figure 1i). The cells elongated again into a cylindrical shape. When viewed under a light microscope, they typically contained a single parietal chloroplast (Figure 1a–i). The centrally located nucleus was surrounded by a chloroplast identified by the wavelength-specific fluorescence of chlorophyll a and DAPI. The chloroplast and nucleus were colored blue and red, respectively (Figure 1j,k). In previous studies, a TEM image analysis revealed the organelle morphology of lipid bodies, starch granules, thylakoids, and mitochondria in the genus *Picochlorum* [21,25,29]. Lipid bodies, which were stained with Nile Red and are indicated by white arrows, exhibited a light green hue (Figure 1l,m). The lipid body was distributed ubiquitously within the cell, with a notable concentration around the nucleus of the stained cells. An observation of DAPI-stained nuclei using fluorescence microscopy revealed that, even in elongated and cylindrical cells, a single nucleus was present and two nuclei were observed just before cell division (Figure 1j–m).

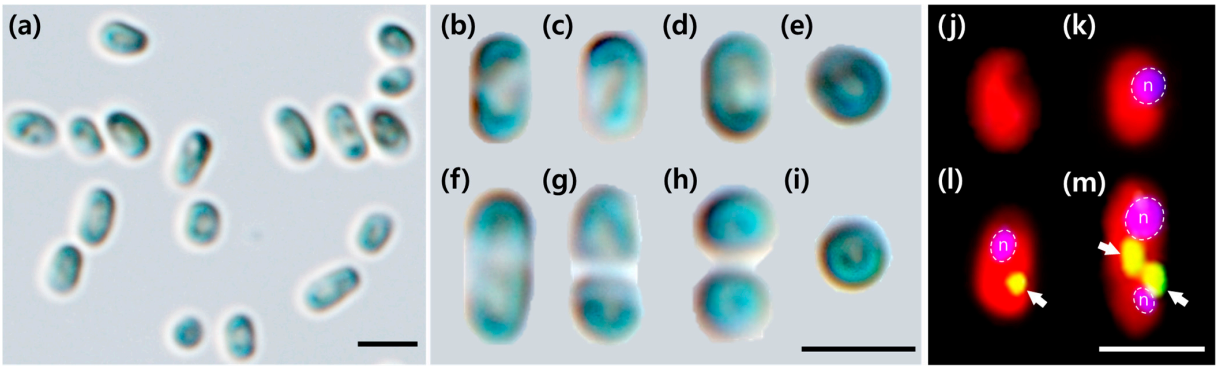

Figure 1. Light and fluorescence micrographs of *Picochlorum* sp. (KCTC AG61293) vegetative cells (**a**). The micrographs are the front view (**b**–**d**) and vertical view (**e**) of the vegetative cell. The micrographs are elongated cell (**f**), dividing cell; binary fission (**g**), and cells immediately after division (**h**), spherical-shaped cell (**i**). The cell stained with DAPI reveals the location of the nucleus (n) and the chloroplast (red) arranged around it. The presence of neutral lipids is indicated by the light green fluorescence (**l**,**m**) observed when Nile Red is present (white arrows), while the red fluorescence represents chlorophyll auto-fluorescence (**j**–**m**). Scale bars = 2 µm.

While invisible under a light microscope, FE-SEM enables the observation of the morphology of the cell surface (Figure 2a–c). The cells elongated and divided from the center to form a spherical shape, consistent with observations from light microscopy (Figure 2d–f). Notably, *Picochlorum* sp. (KCTC AG61293) assumed a spherical shape immediately after division, undergoing binary fission. This may be affected by culture conditions, but based

on the observation of cells at various growth stages using light, fluorescence, and electron microscopy and the ratio of cell length to width (1.3–1.6, including spherical cells), *Picochlorum* sp. (KCTC AG61293) had a cylindrical shape. This shape was confirmed as the basic shape. Under good conditions, divisions occurred continuously, leading to the presence of cells that were more than triple the average length.

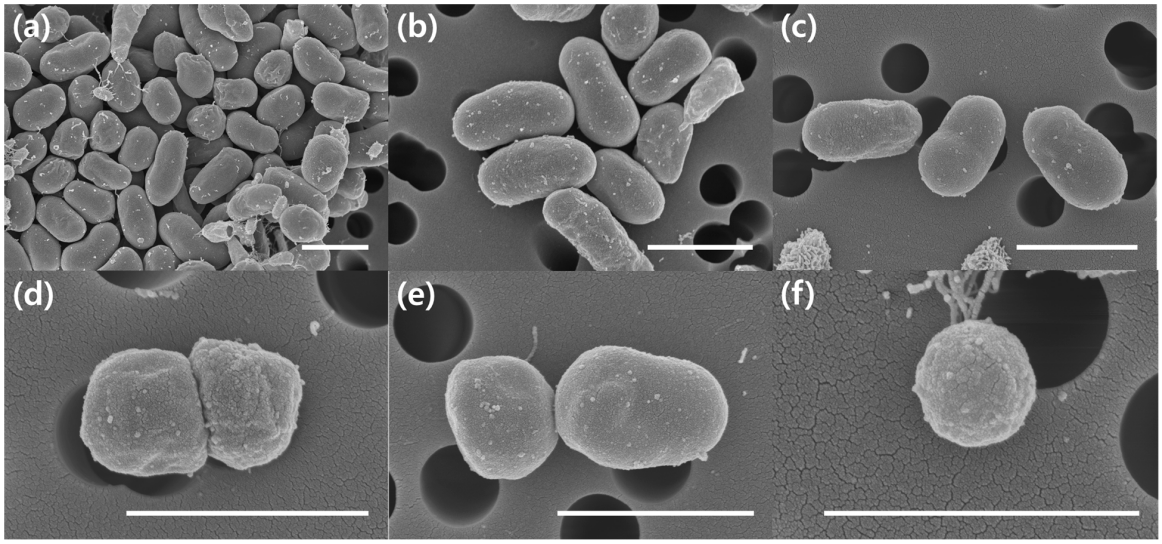

Figure 2. SEM images of *Picochlorum* sp. (KCTC AG61293) display varying shapes (**a**–**c**). The micrographs depict a cell undergoing division; binary fission (**d**), cells post-division (**e**), and a small spherical cell (**f**). scale bars = 2 µm.

Picochlorum sp. (KCTC AG61293) was isolated from seawater, along with other species of the genus *Picochlorum*. The taxonomy of unicellular coccoid green algae, including *Picochlorum* species, has been a complicated and hotly debated issue, due to debates about discriminating morphological or physiological characters [25,48]. While molecular phylogenetic analysis is crucial for their taxonomy, morphological analysis remains indispensable [27,36,49]. The taxonomy of green algae can be improved by combining ecological and DNA sequence data. This provides valuable insights into their structure and morphology, aiding in accurate classification and understanding [36,49,50]. Although all species within the genus *Picochlorum* share some common morphological features, they can be differentiated based on aspects such as cell dimensions, shape, and asexual reproductive patterns [26,27,51]. *Picochlorum* sp. (KCTC AG61293) undergoes an annular contraction-type division, in which the cell elongates and divides into two cells with an annular contraction in the middle, known as binary fission (Figures 1 and 2), similar to the cell division of *Nannochloris bacillaris* [25,26,52]. Binary fission is the distinctive reproduction type of the genera *Nannochloris* and *Gloeotila* [26,27]. The type species of the genus *Picochlorum*, *Picochlorum oklahomensis* (980625–4A), features a size of 2 µm and a length to width ratio of 1.15–1.2. Notably, it reproduces through autosporulation, distinguishing it as a distinct species from *Picochlorum* sp. (KCTC AG61293) [25]. Similarly, *P. atomus* (CCAP 251/7) and *P. maculatum* (CCAP 251/3) differ from *Picochlorum* sp. (KCTC AG61293), in that they reproduce through autosporulation [25–27,53,54]. *P. eukaryotum* (Mainz l) exhibits size ranges of 0.8–1.5 µm in width and 1.1–2.2 µm in length, and the SEM observation showed that the cells are roundish or spherical with an average diameter of 1.5 µm [55]. This contrasts with the size and shape of *Picochlorum* sp. (KCTC AG61293). *P. oculatum* (UTEX 1998) is currently regarded as a synonym of *Nannochloropsis oculata* (Droop D. J. Hibberd), characterized by a subspherical shape and a diameter of 2–4 µm, differing in size and shape from *Picochlorum* sp. (KCTC AG61293) [56]. *P. costavermella* (RCC4223) is a small ovoid single cell of 1–2 µm in length and 1 µm in width. While this species shares a comparable size with *Picochlorum* sp. (KCTC AG61293), it distinguishes itself by having a

distinct ovoid shape [48,57]. A morphological analysis demonstrated that *Picochlorum* sp. (KCTC AG61293) exhibited morphological disparities with all six taxonomically identified *Picochlorum* species, including *Nannochloropsis oculata*.

3.2. Molecular Phylogeny

The phylogenetic placement of *Picochlorum* sp. (KCTC AG61293) was deduced from the ssu sequence (GenBank accession number: OR854634) obtained in this study (Figure 3). Both Bayesian inference and Maximum Likelihood (ML) phylogenies displayed consistent topologies, with the majority of clades receiving strong bootstrap and posterior probability (PP) support. The phylogenetic analysis identified a clade within the phylogenetic group of *Picochlorum* with high statistical support (0.99 PP; 83% ML bootstrap) (Figure 3). *Picochlorum* formed a sister clade with *Nannochloris*, *Marvania*, and other genera, displaying strong support (1.00 PP; 96% ML bootstrap). The ssu data set, comprising 32 sequences from the *Picochlorum* and *Nannochloris* strains, formed a monophyletic group with strong statistical support (1.00 PP; 98% ML bootstrap) and diverged into two distinct groups (Figure 3). In the first group, *P. oklahomensis*, *P. costavermella*, and *P. maculatum* clustered with *Nannochloris* sp. and *Picochlorum* sp. Additionally, *P. atomus* (CCAP 251/7) diverged independently, with good statistical support (0.90 PP; 70% ML bootstrap).

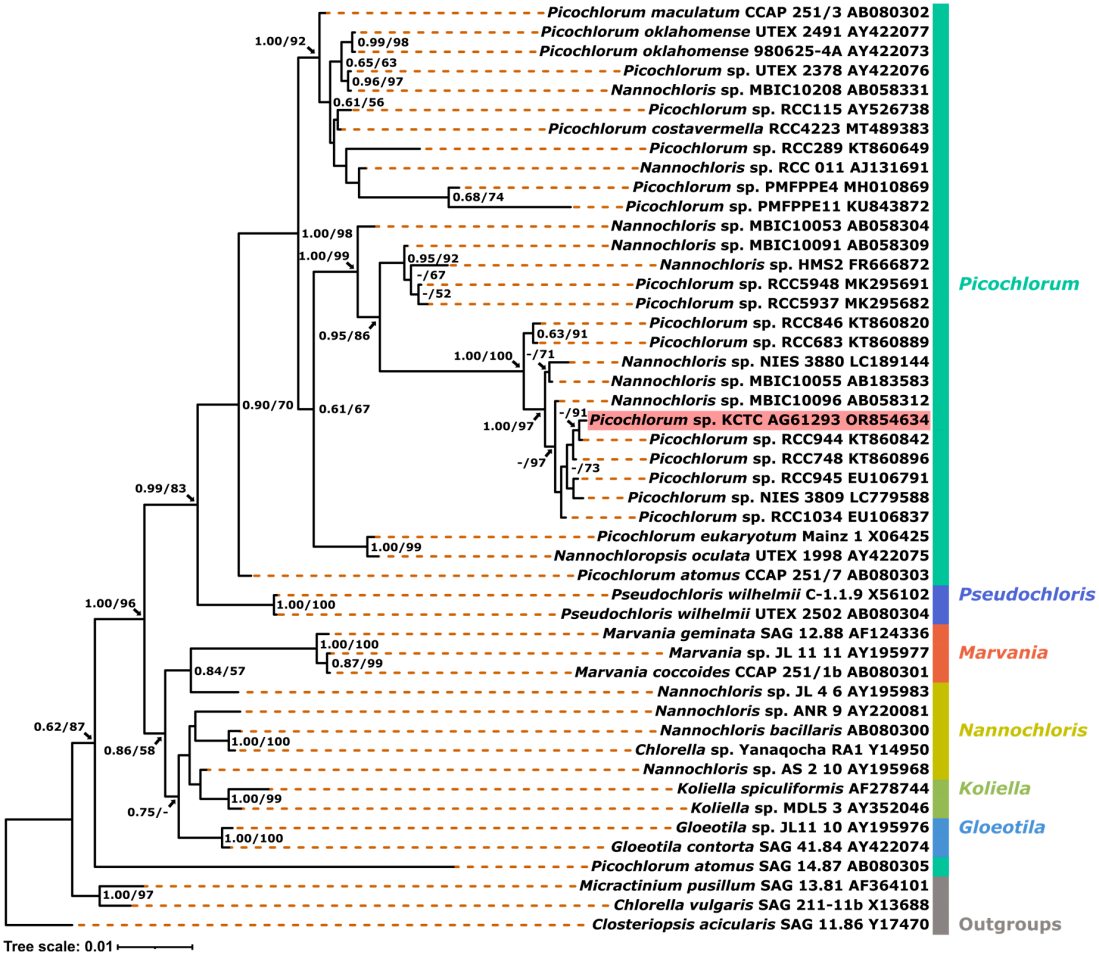

Figure 3. Bayesian inference (BI) tree showing the phylogenetic position of *Picochlorum* sp. (KCTC AG61293) based on ssu sequences. The sequences of *Closteriopsis acicularis*, *Micractinium pusillum*, and *Chlorella vulgaris* were used as outgroups. In the rectangular trees, only bootstrap values greater than 50% and Bayesian posterior probabilities exceeding 0.5 are displayed. The taxon names are indicated followed by the names of the strains and accession numbers of GenBank. The scale bar represents 0.01 nucleotide substitutions per site.

The second group consisted of the genera *Picochlorum* and *Nannochloris*, exhibiting relatively weak statistical support (0.61 PP; 67% ML bootstrap). Within this group, *P. eukaryotum*, *N. oculata* formed a cluster with *Nannochloris* sp. and *Picochlorum* sp. Furthermore, *Picochlorum* sp. (KCTC AG61293) and 10 strains demonstrated high statistical support (1.00 PP; 100% ML bootstrap).

Even minor variations in a sequence can indicate distinct species [48]. The 18S rRNA gene sequence is a dependable marker for identifying new species due to its high conservation. However, it is important to note that relying solely on a single gene marker may not adequately reflect the genetic diversity within a genus. Consequently, the use of multiple gene markers is often more effective in comprehensively understanding phylogenetic relationships [58]. However, for the genus *Picochlorum*, there is a significant lack of sequence information using additional gene markers such as the chloroplast 16S rRNA or ribulose bisphosphate carboxylase (rbcL) gene alongside the 18S rRNA gene marker. In addition, accurate species identification requires genetic information from the same strain. In this study, we also present the chloroplast 16S rRNA gene sequence of *Picochlorum* sp. (KCTC AG61293). The sequence (GenBank accession number: PP109158) was analyzed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) database [59,60], confirming its classification as a chloroplast gene of the genus *Picochlorum*.

The phylogenetic tree constructed from the ssu sequences in this study indicates a close relationship between *Picochlorum* sp. (KCTC AG61293) and other *Picochlorum* species. The tree also highlights the proximity between the genera *Picochlorum* and *Nannochloris*, which is consistent with previous studies [25,27,36,58]. This observed closeness in phylogeny is further supported by shared morphological features, as both genera consist of small, coccoid, and unicellular green algae.

A molecular phylogenetic analysis confirms that *Picochlorum* sp. (KCTC AG61293) is monophyletic and distinct from the six taxonomically named *Picochlorum* species, including *N. oculata*, as shown in the ssu-based phylogeny (Figure 3). This finding is consistent with the results of the morphological analysis conducted in this study. Previous studies have reported that most *Picochlorum* species are autosporulated and have an oval or coccoid shape [25–27,36,51]. This is different from the binary fission and cylindrical shape of *Picochlorum* sp. (KCTC AG61293). The species within the clade nested in *Picochlorum* sp. (KCTC AG61293) have not been given a taxonomical name, suggesting the possibility of a new species of *Picochlorum* based on cell division and cell morphology.

Unfortunately, the current categorization of species within the clade that includes *Picochlorum* sp. (KCTC AG61293) is inadequate. The morphological characterization of many *Picochlorum* strains is notably deficient, which hinders their correlation with phylogenetic placements [36]. To refine our understanding, comprehensive whole-cell ultrastructural analyses and genomic data integration hold promise. These advancements are expected to contribute substantially to fostering a more nuanced species-level classification for *Picochlorum* sp. (KCTC AG61293) and its counterparts within the broader taxonomy.

3.3. Comparing the Fatty Acid Composition of Picochlorum sp. *(KCTC AG61293) in Relation to Other Species within the Picochlorum Genus*

The analysis revealed the presence of 11 FAME compounds, including 1 internal standard (IS) and 3 unidentified FA peaks (Figure 4). The identified saturated fatty acids (SFAs) encompassed myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), and stearic acid (C18:0). Among the monounsaturated fatty acids (MUFA), the identified compounds were cis-10-pentadecenoic acid (C15:1), palmitoleic acid (C16:1), ginkgolic acid (C17:1), and oleic acid (C18:1). The polyunsaturated fatty acids (PUFAs) included linoleic acid (C18:2) and ALA (C18:3n3). The contents of SFA, UFA, MUFA, and PUFA in *Picochlorum* sp. (KCTC AG61293) were 40.40%, 56.77%, 19.00%, and 37.78%, respectively. The primary identified FAs (>10%) were C16:0, C17:1, and ALA. SFAs made up the largest portion of the total FA contents, with C16:0 contributing 36.32%, followed by C18:0 at 2.85%.

ALA accounted for 32.56% of the total FAs. The total FAME contents in *Picochlorum* sp. (KCTC AG61293) was 6.73 ± 0.77%, and the weight of ALA as omega-3 in dry biomass was 21.9 mg/g.

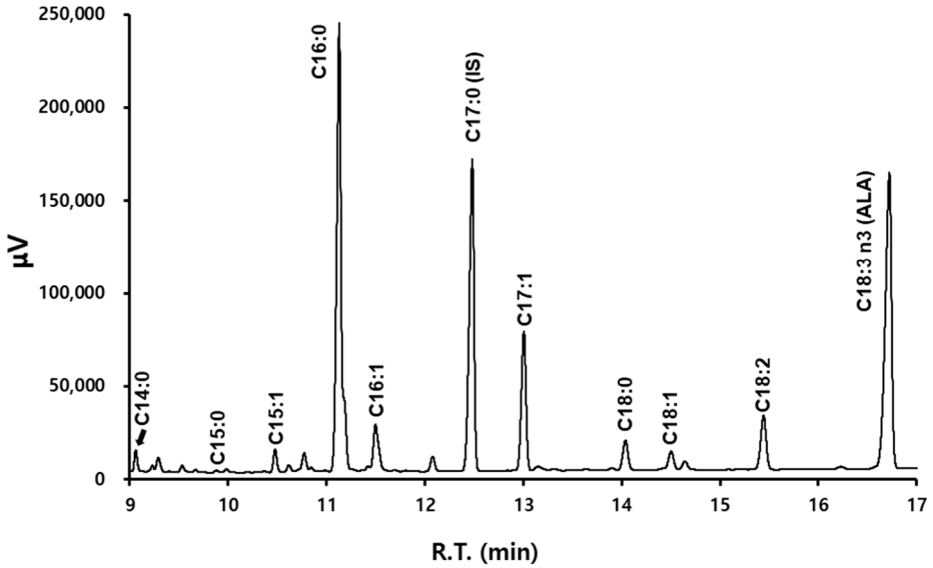

Figure 4. Chromatogram of the FAME composition of *Picochlorum* sp. (KCTC AG61293) on a DB-23 capillary column.

The comparison of the FAME contents in *Picochlorum* sp. (KCTC AG61293) and three related strains revealed a diverse profile of long-chain fatty acids (LCFAs) ranging from C14:0 to C24:1 (Table 1). The C19:0 and C26:0 values shown in Table 1 are taken from a previous study [34]. The total FAME contents in the biomass of *Picochlorum* sp. (KCTC AG61293) were contrasted with those of *Picochlorum* sp. (CTM20019), *P. oklahomensis* (UTEX B 2795), and *N. oculata* (UTEX LB 2164) [34,35]. The FA composition exhibited significant variations among the different *Picochlorum* species, with *Picochlorum* sp. (KCTC AG61293) demonstrating the highest SFA contents (40.40%) and the lowest PUFA contents compared to the other species. The diverse profile of LCFAs in *Picochlorum* sp. (KCTC AG61293), ranging from C14:0 to C24:1, indicates the potential of this strain for biodiesel production [61]. LCFAs are crucial for biodiesel production due to their energy contents and combustion properties. Remarkably, the ALA content as omega-3 of *Picochlorum* sp. (KCTC AG61293) was considerably superior to that of the other species. Specifically, *Picochlorum* sp. (KCTC AG61293) exhibited the highest composition of C16:0 and ALA at 36.32% and 32.56%, respectively, with SFAs contributing the highest contents at 40.40%, especially C16:0, which is a key component of high-acid oil biodiesel [13]. As the carbon chain length in biodiesel fatty acid esters increases, the combustion quality of diesel fuel, as measured by cetane number, improves, resulting in better combustion properties [62]. Certain algae species known to produce significant amounts of C16:0 are considered to be promising biodiesel feedstocks [63]. Moreover, ALA is recognized for its role in maintaining a normal heart rhythm and pumping function, which can lower the risk of heart disease [64]. Diets high in ALA have been linked to a decrease in heart disease, blood pressure, triglycerides, arterial plaque, and myocardial infarction [14,64]. As the body cannot synthesize ALA, it must be obtained through the diet [14]. In contrast to *Picochlorum* and *Nannochloropsis*, which showed negligible omega-3 fatty acid compositions apart from ALA, a minimal amount of DHA was detected in *N. oculata* [34]. However, no EPA or DHA was detected in *Picochlorum* strains. Consequently, the omega-3 contents in each strain were almost equivalent to the ALA contents. This is in contrast to the discoveries made in *Nannochloropsis* species, which are recognized as having the most potential to be photoautotrophic sources of EPA for human intake [65–67]. However, this may be due to differences in species, strain, or culture

conditions [6,68]. Tran et al. [69] reported an increase in DHA contents in a *Picochlorum* strain due to variations in medium composition and culture conditions. However, most studies on lipid production in *Picochlorum* are still lacking and, in most studies, it is difficult to find traces of EPA or DHA in the lipid data of *Picochlorum* strains [29,34,35,70]. It is challenging to determine whether this is attributed to the absence of long-chain fatty acid synthase enzymes or issues with culture conditions or phases. The fatty acid profile of microalgae can indeed be influenced by culture conditions. For example, variations in nutrient availability can significantly affect the production of specific fatty acids [71,72]. Similarly, light conditions, including intensity and photoperiod, can affect microalgal growth and the levels of lipids, carotenoids, and fatty acid composition [73–75]. Therefore, the optimization of these conditions is critical for maximizing fatty acid production. Our results suggest that *Picochlorum* sp. (KCTC AG61293) boasts a distinctive fatty acid profile characterized by elevated levels of SFAs and ALA, positioning it as a promising candidate for biofuel production and various industrial applications [76–78]. However, there are several challenges to scaling up microalgae cultivation for industrial applications. These include the need for low-cost, standardized, industrial-scale microalgae production equipment, the optimization of production processes, and addressing issues related to biological contaminants and microalgae compounds [79–82]. In addition, safety and regulatory issues are major concerns, and extensive research is still needed to make microalgae a commercial success [6,80]. When comparing different microalgae strains for biofuel production, factors such as lipid productivity and fatty acid profile are typically evaluated [83,84]. However, it is important to note that the optimal conditions for lipid production and key high-value metabolites can vary significantly among different microalgae species [6,85]. Nevertheless, further investigations are imperative to comprehensively understand the metabolic pathways and environmental factors influencing the fatty acid composition of this strain. The optimization of cultivation conditions is also crucial for maximizing fatty acid production [6,28,35]. While our study provides valuable insights into the fatty acid profile of *Picochlorum* sp. (KCTC AG61293), further research is needed to fully understand the influence of culture conditions on fatty acid production, to address the challenges associated with scaling up microalgae cultivation, and to compare the performance of this strain with other high-lipid-producing microalgae strains.

Table 1. FA composition comparison between *Picochlorum* sp. (KCTC AG61293) and related *Picochlorum* and *Nannochloropsis* strains.

	% of Total Fatty Acids (C14:0~C26:0 *)			
Taxonomic Group	**Trebouxiophyceae**			
Fatty Acids/Species	***Picochlorum* sp.**	***Picochlorum* sp. ****	***P. oklahomensis* ***	***Nannochloropsis oculata* ***
Strain	**KCTC AG61293**	**CTM20019**	**UTEX B 2795**	**UTEX LB 2164**
C14:0	1.04	nd	0.64	0.34
C15:0	0.19	nd	0.33	0.02
C15:1	1.66	nd	nd	nd
C16:0	36.32	29.00	23.81	19.46
C16:1	3.68	1.50	8.2	2.02
C16:2	nd	8.50	6.93	2.15
C16:3	nd	11.00	nd	1.67
C17:1	11.37	nd	nd	0.07
C18:0	2.85	nd	1.49	0.81
C18:1	2.28	nd	13.85	12.79
C18:2	5.21	23.50	26.19	10.09
C18:3 n-3 (ALA)	32.56	26.60	13.52	25.78
C18:3 n-6	nd	nd	nd	5.26
C19:0 *	nd	nd	nd	0.19

Table 1. *Cont.*

	% of Total Fatty Acids (C14:0~C26:0 *)			
Taxonomic Group	**Trebouxiophyceae**			
Fatty Acids/Species	***Picochlorum* sp.**	***Picochlorum* sp. ****	***P. oklahomensis* ***	***Nannochloropsis oculata* ***
Strain	**KCTC AG61293**	**CTM20019**	**UTEX B 2795**	**UTEX LB 2164**
C20:0	nd	nd	0.10	0.23
C20:1 n-9	nd	nd	0.15	0.10
C20:2 n-6	nd	nd	0.05	1.29
C20:3 n-6	nd	nd	nd	nd
C20:3 n-3	nd	nd	nd	0.09
C20:4 n-6	nd	nd	nd	nd
C20:5 n-3 (EPA)	nd	nd	nd	nd
C22:0	nd	nd	0.06	0.13
C22:1 n-9	nd	nd	nd	0.15
C22:2	nd	nd	nd	0.01
C22:6 n-3 (DHA)	nd	nd	nd	0.18
C24:0	nd	nd	0.01	0.12
C24:1	nd	nd	nd	nd
C26:0*	nd	nd	0.55	nd
unidentified FAs	2.83	-	4.12	17.06
SFAs	40.40	29.00	26.99	21.30
MUFAs	19.00	1.50	22.20	15.13
PUFAs	37.78	69.60	46.69	46.51
UFAs	56.77	71.10	68.89	61.64
Total n-3	32.56	26.60	13.52	26.05

nd: not identified; Other FAs: total of detected but unidentified fatty acids; SFAs: total sum of saturated fatty acids; MUFAs: total sum of monounsaturated fatty acids; PUFAs: total sum of polyunsaturated fatty acids; UFAs: total sum of unsaturated fatty acids; n-3: Omega-3; n-6: Omega-6; n-9: Omega-9. Data taken from Zhu et al. (2013) * [34], Dahmen et al. (2014) ** [35].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse12020245/s1, Table S1: Algal isolates included in the phylogenetic analyses, using names as they currently apper in culture collections and/or Genbank.

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