

Article

Euchlorocystis marina sp. nov. (Oocystaceae, Trebouxiophyceae), a New Species of Green Algae from a Seawater Shrimp Culture Pond

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Abstract: Oocystaceae is a cosmopolitan family of green algae with distinct morphology and ultrastructure. Most of the reported species in this family are freshwater species, and there are few marine species reported. In this study, we describe a new marine species of Oocystaceae, *Euchlorocystis marina* sp. nov. based on material collected from a seawater shrimp culture pond in Zhanjiang, China. An integrative approach, including phylogenetic analyses of 18S rDNA, light microscopy, and transmission electron microscopy, was used for the taxonomic study of the strains. Morphological observation results showed that it has a multilayer thick cell wall, multiple pyrenoids in the chloroplast, and usually 2–16 cells forming a colony in the extended mother cell wall. These features are morphologically similar to the genus *Euchlorocystis* and are distinguished from other taxa of the family Oocystaceae. The 18S rDNA phylogenetic trees revealed that the strains and *Euchlorocystis subsalina* formed an independent clade in Oocystaceae with robust support. However, horseshoe-shaped chloroplasts and rounder cells morphologically distinguished it from *Euchlorocystis subsalina*. Apart from the morphology, the direct comparison of sequences also supported that they were distinct species. The discovery and description of the new species enriches the marine species record of the family Oocystaceae.

Keywords: colonial morphology; Chorophyta; 18S rDNA; pyrenoid; marine species



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1. Introduction

Oocystaceae is a cosmopolitan family of green algae with distinct morphology and ultrastructure. It has formed unique and complex biological characteristics and ecological environment adaptability in the course of long-term evolution. Some Oocystaceae species, owing to their high nitrogen and phosphorus removal ability [1,2], high CO₂-tolerance [3], simple growth requirements, and adaptability to various environmental conditions [4], have shown great potential in improving the aquaculture water environment [1,4,5], wastewater treatment [2,6], carbon emissions [3], and biofuel [7].

Oocystaceae are widely distributed, with records and reports all over the world. The early morphological classification assigned the Oocystaceae family to the Chlorophyceae class [8,9], but molecular studies confirmed that this family was a monophyletic group in the Trebouxiophyceae class [10–13]. Štenclová et al. [14] conducted a molecular phylogenetic study on 54 Oocystaceae species in 2017, and divided Oocystaceae into three subfamilies and five clades. Recently, five new genera were defined in the family Oocystaceae, including *Planctonemopsis* [15], *Quadricoccopsis* [16], *Euchlorocystis*, *Densicystis* [17], and

Reticulocystis [18]. Currently, 96 genera and 304 species of Oocystaceae have been recorded in AlgaeBase [19]. However, few marine species of this family have been recorded.

As a new member of the Oocystaceae family, the genus *Euchlorocystis* was defined by Liu et al. [17] in 2018. The genus *Euchlorocystis* has multiple pyrenoids, distinguishing it from *Oocystis*. It also has a multilayered cell wall and trough-shaped chloroplast, which is different from the *Oonephris* genus. In addition, the cell size of *Euchlorocystis* and the number of chloroplasts per cell were used as the main morphological markers that distinguish the genus *Eremosphaera*. Currently, only one taxonomically accepted species of *Euchlorocystis* is listed in AlgaeBase [19]. In this study, we isolated a new strain of algae from a mariculture pond of shrimp in Zhanjiang, Guangdong Province, China, and successfully cultured it in the laboratory. Morphological and phylogenetic analyses identified this strain as a new species, *Euchlorocystis marina* sp. nov., belonging to the Oocystaceae (Trebouxiophyceae, Chlorophyta). The results will increase the understanding of the genus *Euchlorocystis* and enrich records of marine species of Oocystaceae.

2. Results

2.1. Morphological Observations

Cells are solitary or in 2–16 cell colonies within a thin, hyaline mucilaginous envelope or within an expanded mother cell wall (Figure 1). Cells are round, oval, or slightly reniform, ranging in size from 11.3 to 16.6 μm long and 6.3 to 10.3 μm wide. Cell walls are thick, sometimes stratified (Figure 1b,c), and smooth, without thickening polars. Mother cell walls are extended according to the number and arrangement of daughter cells and are usually an irregularly round shape, with or without tapered thickening ends (Figure 1b,d). The number and shape of chloroplasts are difficult to discern on the microscopic level. When observed with TEM, a horseshoe-shaped chloroplast was observed, which occupied most of the cell volume (Figure 2a,d). Asexual reproduction was by 2–4 autospores released by a rupture of the mother cell wall. These autospores could be propagated again independently inside the mother cell (Figure 1e,g). The asynchronous cell division led to the size difference between the daughter cells (Figure 1i) and the formation of multilayer mother cell walls (Figure 2e,f).

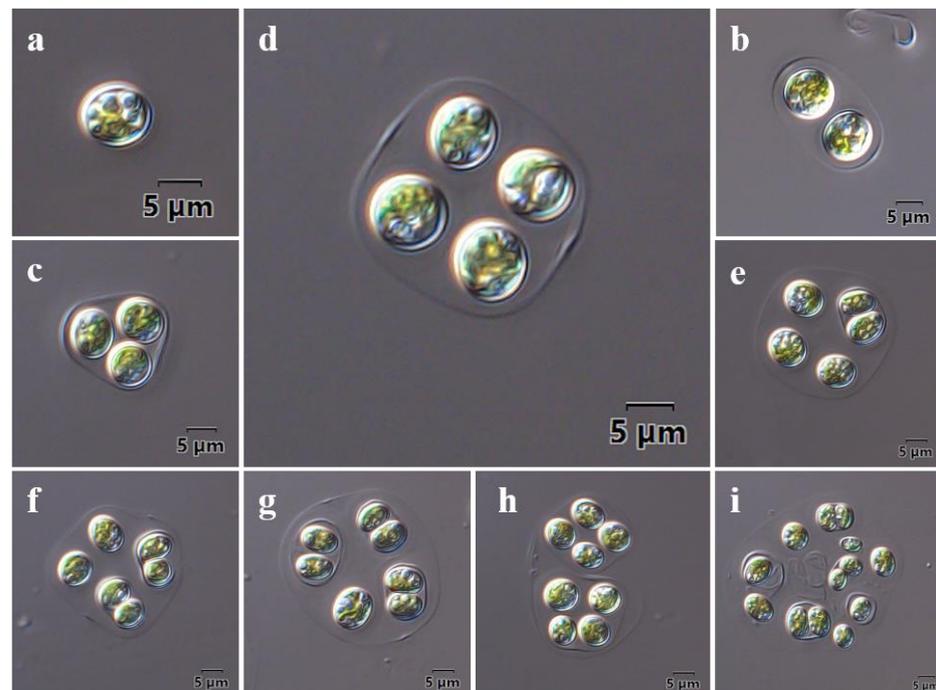


Figure 1. Light microscopy of *Euchlorocystis marina*. (a) Solitary cell; (b) 2-celled colony; (c) 3-celled colony; (d) 4-celled colony; (e) 5-celled colony; (f) 6-celled colony; (g) 7-celled colony; (h) 8-celled colony; (i) 13-celled colony.

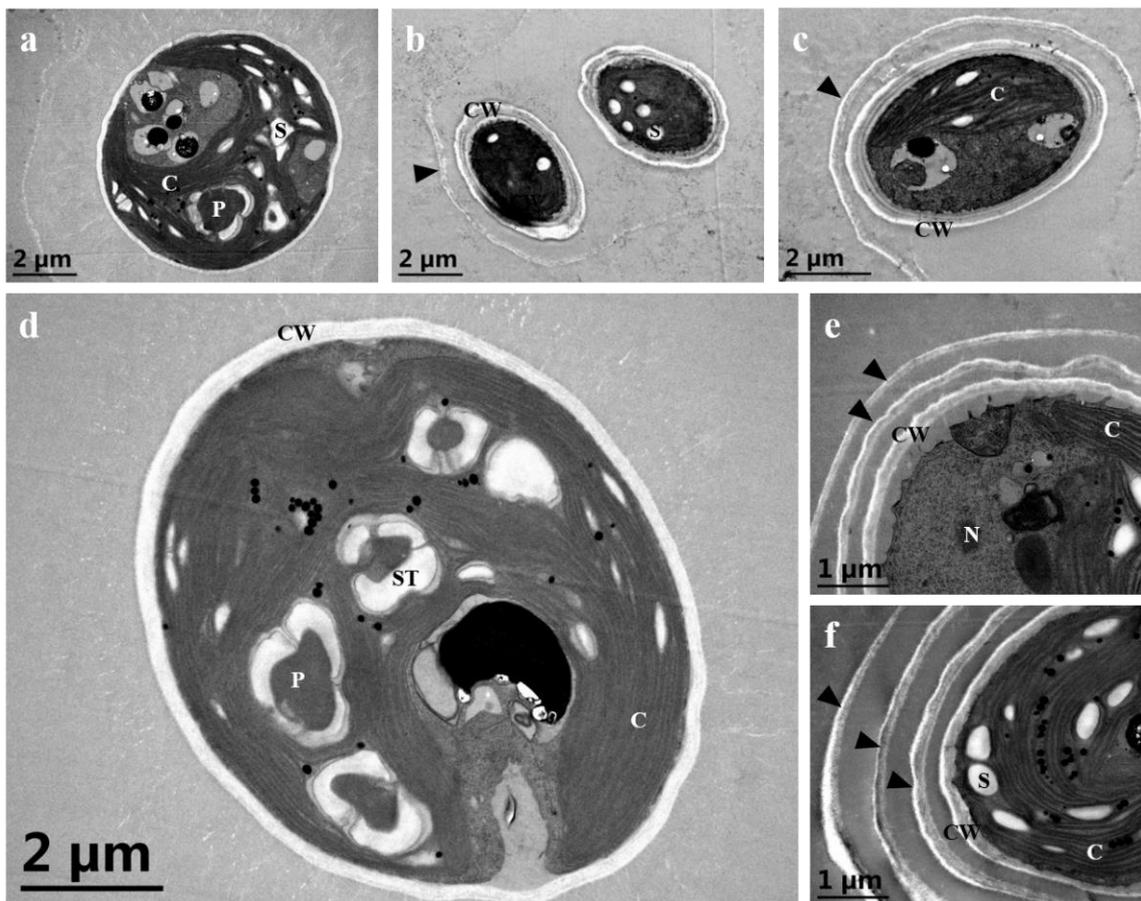


Figure 2. Transmission electron microscopy of *Euchlorocystis marina* sp. nov. (a,d) Mature cells; (b,c) Young cells; (e,f) Ultrastructure of multilayer mother cell wall. (CW = cell wall; C = chloroplast; P = pyrenoid; S = starch grains; ST = starch sheath; N = nucleus). The mother cell walls are indicated by black arrow.

When observed with TEM, 1–4 pyrenoids were observed in the mature cells of *Euchlorocystis marina* (Figure 2a,d). The pyrenoid was surrounded by a starch sheath composed of 2–4 starch plates, and the pyrenoid matrix was homogenous (Figure 2a,d). However, in some young cells, pyrenoids were not observed (Figure 2b,c).

2.2. Molecular Phylogeny

Sequencing of the 18S rDNA of the new strain produced a 1720 bp sequence, introns were excluded. The final 18S rDNA sequences alignment of 29 taxa with 1633 base positions were used for the phylogenetic tree construction, and *Prasiola mexicana* (Prasiolaceae) was chosen for the outgroup. The ML and NJ analyses yielded a similar topology and only the ML tree was presented in Figure 3. The 18S rDNA phylogenetic trees consistently recovered that the newly isolated strain and *Euchlorocystis subsalina* formed an independent clade with high support (99/100 for ML/NJ) (Figure 3). Direct comparison showed that the 18S rDNA of the new strain had 41 differences (97.5% identity) to *Euchlorocystis subsalina* (Table 1).

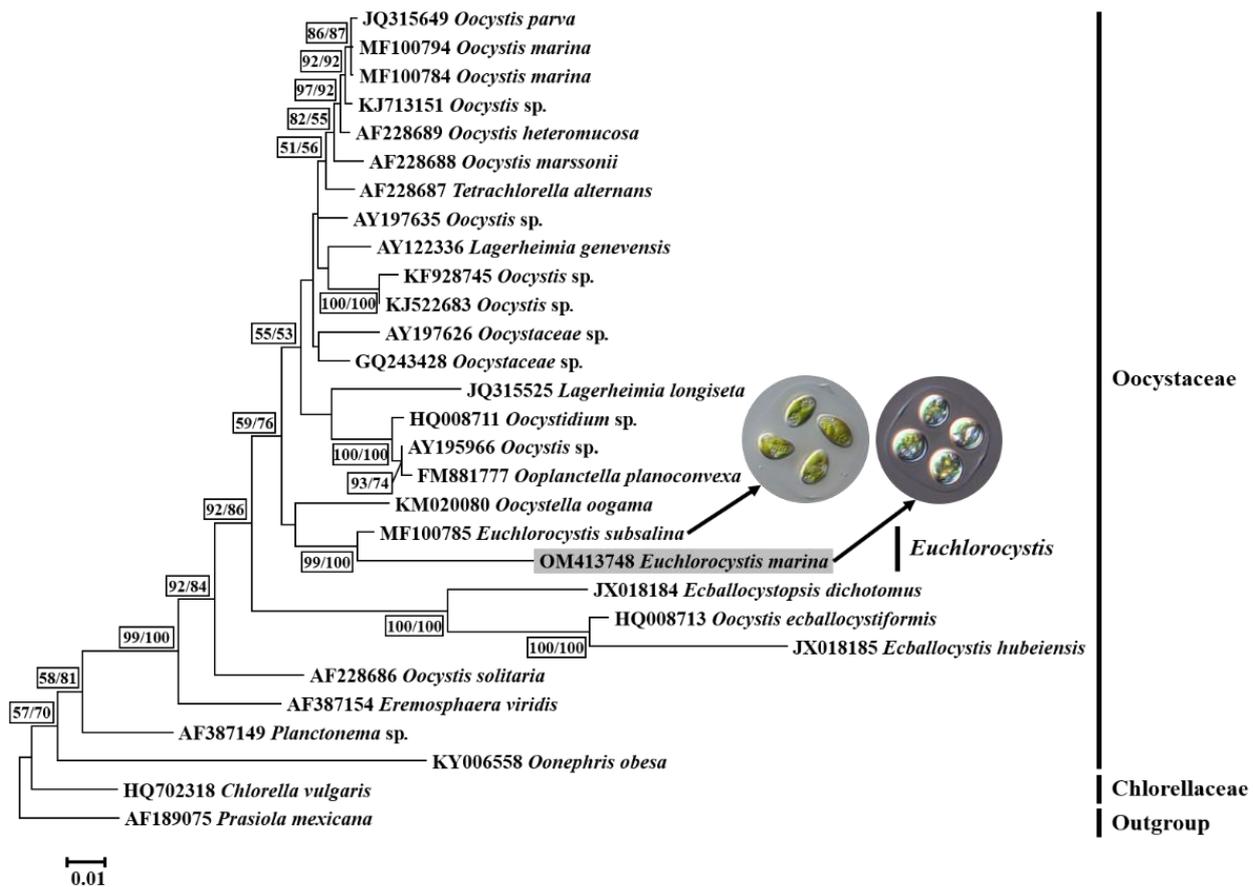


Figure 3. Maximum likelihood tree of 18S rDNA sequences. Bootstrap support from ML and NJ posterior probabilities are presented on the nodes. The sequence obtained in this study is shaded gray. The cell morphology picture of *Euchlorocystis subsalina* was from Liu et al. [17].

Table 1. Direct comparison of 18S rDNA sequences of *Euchlorocystis marina* (bold) with some species of the Oocystaceae family. Numbers below the diagonal indicate nucleotide substitutions between a pair of sequences and those above the diagonal are percent identities. Black boxed area indicates the high sequence similarities between the two closest related species.

Positions Compared: 1633.	Accession Number	<i>Eremo. viridis</i>	<i>Ecball. dichotomus</i>	<i>Oocystis. marina</i>	<i>Euchloro. subsalina</i>	<i>Euchloro. marina</i>	<i>Oocystella. oogama</i>	<i>Oonephris. obesa</i>
<i>Eremosphaera viridis</i>	AF387154	—	90.0%	94.6%	94.5%	92.6%	93.9%	87.6%
<i>Ecballocystopsis dichotomus</i>	JX018184	163	—	90.4%	90.3%	88.1%	90.6%	82.7%
<i>Oocystis marina</i>	MF100784	88	157	—	96.7%	94.7%	95.8%	86.6%
<i>Euchlorocystis subsalina</i>	MF100785	90	158	54	—	97.5%	95.8%	86.8%
<i>Euchlorocystis marina</i>	OM413748	121	194	87	41	—	94.1%	84.6%
<i>Oocystella oogama</i>	KM020080	100	154	69	69	96	—	86.5%
<i>Oonephris obesa</i>	KY006558	202	283	219	216	251	220	—

3. Discussion

Members of the family Oocystaceae are common in freshwater, but they are rarely found in seawater. Currently, only a few marine species in this group are taxonomically accepted, such as *Oocystis submarina*, *Oocystis marina*, and *Euchlorocystis subsalina*. Some other species, due to their extensive adaptation, are also reported to exist in saline or semi-saline conditions [1,20–24], but they are taxonomically considered freshwater species. In this study, a new member of this family was found in a mariculture pond of shrimp in China. An integrative taxonomic study including morphological and phylogenetic analyses identified it as a new species, *Euchlorocystis marina* sp. nov., belonging to the genus *Euchlorocystis* (Oocystaceae).

The genus *Euchlorocystis* was originally erected by Liu et al. [17] in 2018. It has morphology similar to the well-known genus *Oocystis*. As a type of the family Oocystaceae, *Oocystis* has always been controversial in taxonomy. Komárek and Fott [8] listed 28 species with an additional 16 species designated as incompletely described or difficult to identify. Hindák [25] assigned to the genus *Oocystis* only those forms without a pyrenoid and listed eight species for the genus. Those forms included in the genus *Oocystis* with a pyrenoid were classified in the genus *Oocystella* [26]. The chloroplasts of *Euchlorocystis* mature cells have multiple pyrenoids, a feature that easily distinguishes them from *Oocystis*, *Oocystella*, and other Oocystaceae members without or with one pyrenoid. *Oonephris* species were described as having a central pyrenoid and sometimes more than one pyrenoid within the chloroplast [8]. According to Liu et al. [17], *Euchlorocystis* was morphologically different from *Oonephris* in terms of chloroplast shape and layered cell walls. We believe that the asynchronous cell division present in *Euchlorocystis* is also a distinctive feature to distinguish from *Oonephris*. *Eremosphaera* were also described as having more than one pyrenoid [8]. Liu et al. [17] suggested that *Euchlorocystis* and *Eremosphaera* could be distinguished by cell size and the number of chloroplasts per cell. In addition, the number of cells within colonies and whether the division of daughter cells within the mother cell is synchronized can also distinguish *Euchlorocystis* from *Eremosphaera*. In conclusion, the asynchronous cell division may be one of the most distinctive features for distinguishing *Euchlorocystis* from other taxa of the family Oocystaceae.

Euchlorocystis subsalina was originally described by Liu et al. [17] based on the materials collected from a semi-saline lake, Qinghai Lake. It is a type of the genus *Euchlorocystis* and is the only taxonomically accepted *Euchlorocystis* species reported currently. The new species presented here, *Euchlorocystis marina*, was collected from a seawater shrimp culture pond in Zhanjiang, China. It has a multilayer thick cell wall, multiple pyrenoids in the chloroplast, and usually 2–16 cells forming a colony in the extended mother cell wall. These features are morphologically similar to the genus *Euchlorocystis*, and are distinguished from other taxa of the family Oocystaceae. Most importantly, however, the 18S rDNA phylogenetic trees revealed that *Euchlorocystis marina* and *Euchlorocystis subsalina* formed an independent clade in Oocystaceae with robust support. However, horseshoe-shaped chloroplasts and rounder cells morphologically distinguish it from *Euchlorocystis subsalina* (Table 2). Apart from the morphology, the molecular results of this study also supports that they are distinct species. The discovery of *Euchlorocystis marina* may imply more hidden species of the genus *Euchlorocystis* that have yet to be discovered. More new species within this genus should be collected and sequenced in the future.

Taxonomic assessment

Euchlorocystis marina Dong, Li & Huang, sp. nov. (Figure 1a–i)

Diagnosis: Cells round, oval, or slightly reniform, ranging in size from 6.9–12.3 μm long and 4.3–10.7 μm wide. Cell wall thick, sometimes stratified, without thickening polars. Mother cell walls were usually an irregularly round shape, with or without tapered thickening ends. Single horseshoe-shaped chloroplast with 1–4 pyrenoids within mature cells. Asexual reproduction by 2–4 autospores. Daughter cells have asynchronous cell division.

Holotype: Formaldehyde-fixed material was stored at the Algae Resource Development and Culture Environment Ecological Remediation Laboratory of Guangdong Ocean University, Zhanjiang, China, as specimen No. HXH1. The population was partially illustrated here in LM (Figure 1a–i) and TEM (Figure 2a–f).

Reference strain: A living culture was deposited in the Algae Resource Development and Culture Environment Ecological Remediation Laboratory of Guangdong Ocean University, Zhanjiang, China, as specimen GDOU-406.

Type locality: Xuwen seawater shrimp culture pond (20°88' N, 109°74' E; salinity 29‰; water temperature = 30.7 °C; pH = 8.12) in Zhanjiang, China. Water samples were collected in August 2016.

Etymology: The species was named for its habitat of seawater.

Table 2. Morphological comparison between *Euchlorocystis subsalina* and *Euchlorocystis marina*.

Morphology Character	<i>Euchlorocystis subsalina</i>	<i>Euchlorocystis marina</i>
Cell shape	Oval to elongated elliptical with round ends round and no thickenings	Round, oval, or slightly reniform without thickening polars
Cell size	11.3–16.6 µm long and 6.3–10.3 µm wide	6.9–12.3 µm long and 4.3–10.7 µm wide
Cell arrangement	Solitary, 2–16 cell colonies	Solitary, 2–16 cell colonies
Mucilage envelopment	Lemma- to square-shape	Usually irregular round shape, with or without gelled and thickened poles.
Cell wall	Thick, layered.	Thick, layered.
Chloroplasts number	Single	Single
Chloroplast shape	Wide trough shape, parietal	Horseshoe-shaped
Pyrenoids number per chloroplast	2–6	1–4, within mature cells
Cell reproduction	Propagation by 2–4 autospores	Asexual reproduction by 2–4 autospores; There exist the asynchronous division in cell colonies
Reference	[17]	This study

4. Materials and Methods

4.1. Strains and Culture Conditions

Euchlorocystis marina was sampled at a seawater shrimp culture pond (20°88' N, 109°74' E, salinity 29‰, strain GDOU-406) in Zhanjiang, Guangdong Province, China, and maintained in the Algae Resource Development and Culture Environment Ecological Remediation Laboratory of Guangdong Ocean University (Zhanjiang, China). The strain was isolated using the serial dilution pipetting technique (Hoshaw and Rosowski, 1973) until single colonies were obtained. Then, individual colonies were cultivated in a seawater medium (Zhanshui 107–13) at pH = 8.0 with the following nutrient compositions: KH_2PO_4 ($8 \text{ mg}\cdot\text{L}^{-1}$), NaNO_3 ($80 \text{ mg}\cdot\text{L}^{-1}$), ferric citrate ($50 \text{ mg}\cdot\text{L}^{-1}$). The cultures were carried out in a 500 mL Erlenmeyer flask containing 300 mL medium at 25 ± 1 °C and $30 \text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of continuous light (T8 LED lamps, white light).

4.2. Morphological Observation

Microscopic observation and photographing of algal cells were performed using an OLYMPUS BX53 Light microscope.

For ultrastructure, algal cells were collected by centrifugation (3000 rpm, 5 min) and washed three times with PBS buffer. Then, the cells were fixed with 2.5% glutaraldehyde solution for 2 h at 4 °C, and post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 2 h at 4 °C. The fixed materials were dehydrated with a graded acetone series and embedded in Spurr resin. Uranium acetate and lead citrate were used to stain the final sections. The samples' slice preparation and staining were performed by Guangdong Medical University (Zhanjiang, China). The algal slices were examined and photos taken by a JEOL JEM-1400 transmission electron microscope.

4.3. Molecular Analyses

Total genomic DNA of algae was extracted with the DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The PCR cycling conditions were as follows: 5 min initial denaturation at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s; a final extension of 10 min at 72 °C. The 18S rDNA was amplified using Ech118SF (ACTGTGAACTGCGAATGG) and Ech118SR (TAGGTGGGAGGGTTTAGG) primers. The PCR products were sequenced by Shanghai SaiHeng Biotechnology Co., Ltd., Shanghai, China. The assembled sequences were submitted to GenBank under the accession number OM413748.

The new 18S rDNA sequences were aligned with gene sequences downloaded from GenBank of 28 representative species. Phylogenies were estimated by Clustalx 1.8 and Mega 5.0 software with maximum likelihood (ML) and neighbor-joining (NJ) methods. Kimura 2-parameter model was selected as best-fit model. Bootstrap analysis was performed with 1000 replicates of the dataset to estimate statistical reliability.

Author Contributions: Data curation, F.L. and M.D.; methodology, F.L., N.Z., Y.Z. and Q.L. (Qianru Li); investigation, F.L., M.D., Z.Q., Q.L. (Qingsheng Lian) and J.L.; project administration, C.L. and X.H.; resources, C.L. and X.H.; writing—original draft, F.L. and M.D.; writing—review and editing, F.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: The strain used in this study was collected from a seawater shrimp culture pond (20°88' N, 109°74' E; salinity 29‰; water temperature = 30.7 °C; pH = 8.12) in Xuwen, Zhanjiang, China. A living culture and formaldehyde-fixed material were stored at the Algae Resource Development and Culture Environment Ecological Remediation Laboratory of Guangdong Ocean University, Zhanjiang, China. The 18S rDNA sequences of this strain has been submitted to the GenBank under the accession number OM413748.

Conflicts of Interest: The authors declare no conflict of interest.

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