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SYMBIOCHLORUM HAINANENSIS GEN. ET SP. NOV. (ULVOPHYCEAE, CHLOROPHYTA) ISOLATED FROM BLEACHED CORALS LIVING IN THE SOUTH CHINA SEA[1]

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Light/scanning electron/transmission microscopy-based morphological analyses and multiple nucleotide sequences-based molecular phylogenetic analyses are used to identify and assess the phylogenetic position of a new unidentified green alga isolated from bleached corals living in the South China Sea. This new unidentified green alga is a unicellular marine alga and has uninucleate vegetative cells and multiple chloroplasts with a pyrenoid. It can form aplanosporangium covered by cell walls and reproduces by releasing autospore. These features differ substantially from those of the two genera *Ignatius* and *Pseudocharacium*. Those two genera have been accommodated in the Ignatius clade. Nucleotide sequences of the nuclear small subunit ribosomal RNA gene (18S rRNA), internal transcribed spacer 2 of ribosomal RNA gene (ITS2) and ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L, partial) are obtained and compared with published green algal sequences. The results from the morphology, ultrastructure, and multiple nucleotide sequences data support the placement of the new unidentified green alga in Ulvophyceae. This new unidentified isolate is described as *Symbiochlorum hainanensis* gen. et sp. nov., a new sister lineage to the Ignatius clade, Ulvophyceae, Chlorophyta.

Key index words: **18S rRNA; Chlorophyta; *Ignatius*; ITS2; Morphology; *rbcL*; *Symbiochlorum hainanensis*; Ultrastructure; Ulvophyceae**

***Abbreviations*: 18S rRNA, nuclear small subunit ribosomal RNA gene; AP, autospore; Ch, chloroplast; CW, cell wall; ITS2, internal transcribed spacer 2 of ribosomal RNA gene; N, nucleus; P, pyrenoid; V, vacuole**

The Ulvophyceae are dominant algae of Chlorophyta in marine habitats (Cocquyt et al. 2010, Arora et al. 2013), containing eight orders classically distinguished by their life cycles and ultrastructure, and more recently through molecular phylogenetics using a range of markers (Leliaert et al. 2015). Four main cytomorphological types can be distinguished in the Ulvophyceae. The first type comprises green algae with a single nucleus and chloroplast. This is present in genera of uncertain affinity (e.g., *Oltmannsiellopsis*, *Ignatius*) and some early-branching Ulvales and Ulotrichales (Nakayama et al. 1996, Friedl and O'Kelly 2002, Watanabe and Nakayama 2007). The second type has multicellular bodies composed of uninucleate cells. In external morphology, these algae are filamentous or sheet-like. This cytomorphological type is present in most of the Ulvales, Ulotrichales, and Trentepohliales (Cocquyt et al. 2010). The third type is known as the siphonocladous type and has multicellular bodies composed of multinucleate cells as a consequence of uncoupled cell division and mitosis (Motomura 1996). This cytomorphology is typical of the Cladophorales and *Blastophysa*, a genus of uncertain affinity. The fourth type is better known as the siphonous type and is characterized by a single giant tubular cell (Vroom and Smith 2003). It is present in the orders Bryopsidales and Dasycladales (Verbruggen et al. 2009).

Phylogenetic analyses have provided a relatively robust phylogenetic framework of the class Ulvophyceae (Cocquyt et al. 2010). Nearly five clades of green algae within the class Ulvophyceae have been recovered. The Oltmannsiellopsidales+Ulvales-Ulotrichales clade and a clade consisting of the Trentepohliales, Cladophorales, Bryopsidales, and Dasycladales (TCBD clade) have been recovered in phylogenetic analyses inferred from nuclear and chloroplast genes (Cocquyt et al. 2010, Škaloud et al. 2013, Fang et al. 2017). Other smaller clades within the class Ulvophyceae are the Scotinosphaerales, Ignatius, and Blastophysa clades (Cocquyt et al. 2010, Škaloud et al. 2013, Fang et al. 2017). At present, five clades of green algae, including eight orders within the class Ulvophyceae, have been classified based on cytomorphological and phylogenetic analyses (Cocquyt et al. 2010, Škaloud et al. 2013, Fang et al. 2017). Among the five clades, the clade Ignatius is composed of relatively inconspicuous and somewhat neglected taxa confined to terrestrial habitats, including two genera, *Ignatius* and *Pseudocharacium* (Cocquyt et al. 2010).

During the 2015 thermal bleaching period, new unidentified green algal strains were isolated from bleaching coral species (*Platygyra lamellina*, *Porites lutea*, *Favia speciosa*) living in the northern part of

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the South China Sea. Our goal in this study is to describe the morphology and evolutionary relationships of these strains, which turn out to represent a new genus and a new species within the class Ulvophyceae and are closely related to the Ignatius clade. Our approach consists of light/scanning electron/transmission electron microscopy, and molecular phylogenetic analyses based on multiple nucleotide sequences.

MATERIALS AND METHODS

Sampling, isolation, and culture. Healthy and bleached massive coral species *Platygyra lamellina*, *Porites lutea*, and *Favia speciosa* were collected during the 2015 thermal bleaching period from the Luhuitou fringing reef in the northern part of the South China Sea (109.470°–109.489° E, 18.200°–18.217° N). Twelve tissue pieces (~1 cm × 1 cm) from each of the healthy and bleached coral species were collected using a hammer/chisel set, washed three times with filtered seawater, and ground with a pestle to prepare the tissue homogenate. Then, the coral tissue homogenate was further immersed into 100 mL of a ASP8A liquid medium in a 250 mL flask. The ASP8A medium was amended with an KAS antibiotic cocktail (Soffer et al. 2008): 50 $\mu g \cdot mL^{-1}$ kanamycin, 100 $\mu g \cdot mL^{-1}$ ampicillin, and 50 $\mu g \cdot mL^{-1}$ streptomycin. The flasks with the coral tissue homogenate were cultivated at 32°C. All flasks were illuminated with 30 μmol photons $\cdot m^{-2} \cdot s^{-1}$ under a 24 h:0 h L:D regime. Algal cells released from the coral tissues were monitored under a light microscope (magnification up to 1,000×; OLYMPUS, Tokyo Japan). Subsequently, 100 μL of the ASP8A liquid medium with coral tissue homogenate was spread onto a solid ASP8A medium (amended with the KAS antibiotic cocktail) and grown at 32°C with 30 μmol photons $\cdot m^{-2} \cdot s^{-1}$ under a 12 h:12 h L:D regime. Algal colonies on the solid ASP8A medium were picked and transferred into 10 mL of a liquid ASP8A medium in 50 mL flasks. A total of 100 μL of algal culture was spread onto the solid ASP8A medium and incubated under the same conditions. This process was repeated several times to obtain axenic algal strains.

Light, scanning electron, and transmission electron microscopy. Algal cultures in log and stationary phases were sampled for light, scanning electron, and transmission electron microscopy. Light microscopy of algal cells was observed and recorded using a light microscope (CX41, magnification up to 1,000×; OLYMPUS), and the morphology was documented using a Mshot Digital Imaging System (MC50; Mshot, China, Guangzhou). For scanning electron microscopy (SEM), algal samples were centrifuged at 5,000 rpm = 1676*g* for 10 min, washed three times with a filtered Sörensen-sucrose phosphate buffer (pH 7.5), dehydrated in an ethanol series (30%, 50%, 70%, 90%, and 95% for 10 min each, followed by two 30 min changes in absolute ethanol, and finally critical-point dried in a critical-point drying device (Leica EM CPD030, Wetzlar, Germany) using liquid carbon dioxide and ion sputter-coated with gold. Then, the dried cells were observed using a scanning electron microscope (Sirion 200, FEI Corp., Hillsboro, USA). For observations with a transmission electron microscope (TEM), algal samples were centrifuged at 5,000 rpm = 1676*g* for 10 min and washed three times with a filtered Sörensen-sucrose phosphate buffer (pH 7.5). Samples were further fixed for 1 h at room temperature in 1% OsO_4, dehydrated in ethanol and embedded in Spürr resin. Sections were cut with a cryo-ultramicrotome (UC6-FC6, Leica, Wetzlar, Germany). Ultra-thin sections (~70 nm) were mounted on formvar-coated alphanumeric grids, counterstained with 2% uranyl acetate in 50% ethanol, and observed with a 120-kV biology transmission electron microscope (Tecnai G2 Spirit Bio twin, FEI Corp., Hillsboro, USA).

DNA sequencing and phylogenetic analyses. The total DNA of algal cultures was isolated with the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The 18S rRNA, *rbc*L, and ITS2 nucleotide sequences were amplified using universal primers (An et al. 1999, Paul et al. 2000, Duff et al. 2008). The PCR amplifications were carried out according to the instructions of the 2× Pfu PCR master mix (Tiangen, Beijing, China). Amplification products were purified (PCR cleanup kits; Tiangen) and sequenced (in both directions using PCR primers) by Sanger sequencing. Sequences were further aligned with sequences available from GenBank (www.ncbi.nlm.nih.gov) by ClustalW in MEGA 6.06 (Tamura et al. 2013). The consensus sequences were submitted to GenBank (18S rRNA,ITS2, and *rbc*L with accession numbers of MH061387, MH061388, and MH061314, respectively). The evolutionary models that fit best for all the datasets (separated according to their genes) were calculated using the program JModelTest version 2.1.7 (Darriba et al. 2012). For the Phylogenetic tree analyses of nucleotide sequences data were computed using two different methods: the Bayesian inference (BI) and maximum likelihood (ML). Analyses were done with a GTR+I+G model. For BI, the chain was run for 10 million generations, saving every 1,000th generation in MrBayes version 3.2.6 (Huelsenbeck and Ronquist 2001). For ML, we performed 1,000 replicates of the rapid bootstrapping algorithm in MEGA version 6.06. (Tamura et al. 2013).

RESULTS

The different algal strains isolated from bleached corals are morphologically similar, and the sequences of 18S rRNA, ITS2, and *rbc*L of those algal strains are identical, respectively; thus, the new unidentified algal strain from bleached *Porites lutea* is used for light/scanning electron/transmission electron microscopy, and molecular phylogenetic analyses based on multiple nucleotide sequences in order to avoid information redundancy. Based on the morphological and molecular data described below, the new unidentified algal strain is identified as a new genus and species, and forms a sister branch to Ignatius clade, Ulvophyceae.

***Symbiochlorum* S. Q. Gong & Z. Y. Li gen. nov.**

Description

Morphological aspects. In the log growth phase, the vegetative cells of this new green alga are usually light green (Fig. 1a), with a visible cell wall (Fig. 1, a, d–f), vacuole (Fig. 1, d and e), nucleus (Fig. 1, a, d, and e), multiple chloroplasts with a pyrenoid (Fig. 1, a, d–f), and are spherical and unicellular, with diameters that ranged from 5 to 12 μm (Fig. 1). The matrix of pyrenoid is covered by starch segments (Figs. 1, a and d, 2e). Clearly, sporic reproduction is observed in the stationary growth phase cultures. The aplanosporangium is observed, which is light green (Fig. 2a) and is spherical with diameters that ranged from 10 to 20 μm (Fig. 2, a, b, and d). The aplanosporangium contains many autospore (Fig. 2c). No zoospores are observed. The aplanosporangium contains chloroplast fragments (Fig. 2, d and e) with a pyrenoid

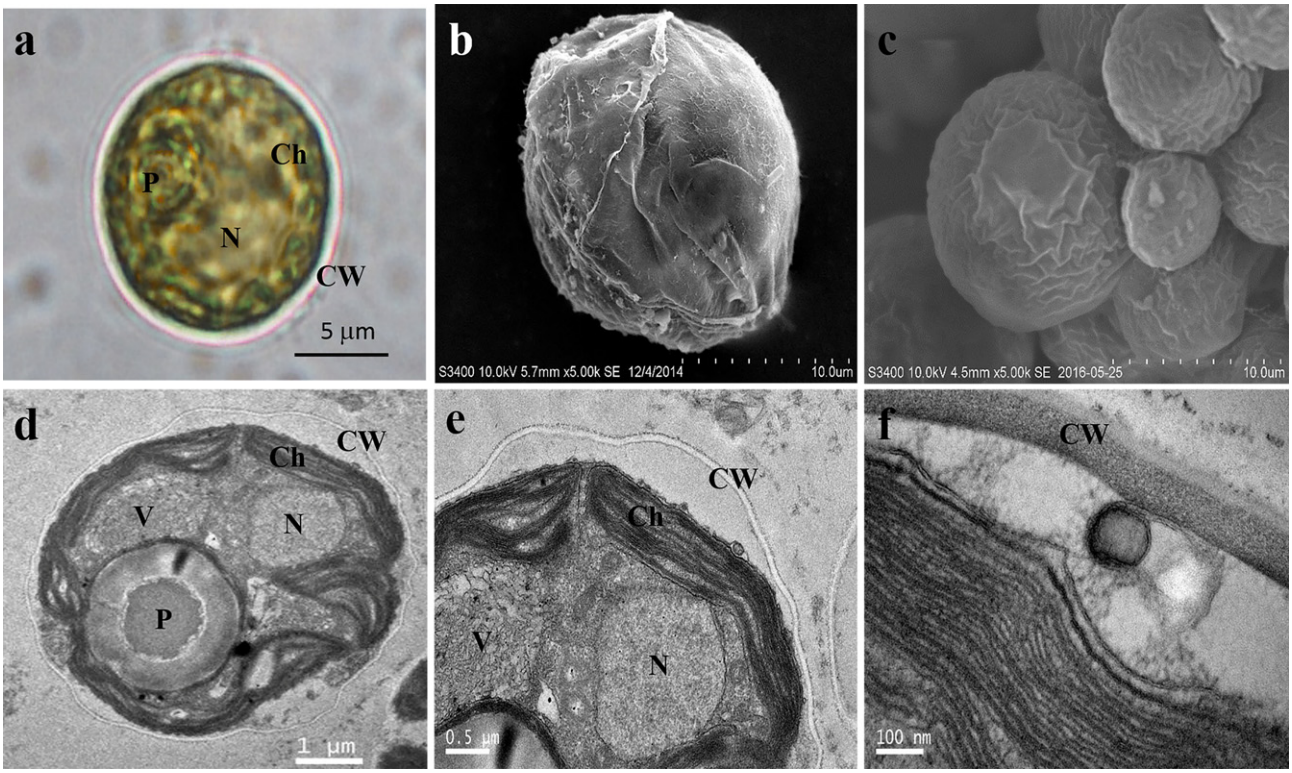

FIG. 1. Light, scanning electron, and transmission electron microscopic presentations of vegetative cells of *Symbiochlorum hainanensis*. (a) Light microscopy. (b) SEM-single cell. (c) SEM-multiple cells with different diameters. (d) TEM, scale = 1 μm. (e) TEM, scale = 0.5 μm. (f) TEM, scale = 100 nm. P, pyrenoid; N, nucleus; V, vacuole; Ch, chloroplast; CW, cell wall. [Color figure can be viewed at wileyonlinelibrary.com]

(Fig. 2, a, d, and e). The nucleus in aplanosporangium becomes obscured. The release of spores by fracture of the cell wall of aplanosporangium (Fig. 2c) is observed. The released spores are elliptical and 1–3 μm in diameter (Fig. 2c). The cell walls of vegetative cells and aplanosporangium lack surface structures such as spines or scales (Figs. 1b and 2b) and the cell walls vary in thickness between vegetative cells (~100 nm; Fig. 1f) and aplanosporangium (~400 nm; Fig. 2f).

Sequence analysis. All phylogenetic analyses of 18S rRNA, ITS2, and *rbc*L nucleotide sequences are carried out using the BI and ML methods under the GTR+I+G model.

In our analysis, a hierarchical strategy is used. First, we included 18S rRNA nucleotide sequences of nine classes of the Chlorophyta sensu Marin (2012), in order to investigate the relative position of this new green alga at the class level. In all trees obtained by BI or ML analysis, this green alga falls within the class of Ulvophyceae, with high Bayesian posterior probabilities of 0.98 and ML bootstrap values of 95%, respectively (Fig. S1 in the Supporting Information). Second, nucleotide sequences of 18S rRNA, ITS2, and *rbc*L of eight orders of Ulvophyceae sensu Cocquyt et al. (2010) and Škaloud et al. (2013) are included. In all the trees (Fig. 3, Figs. S2 and S3 in the Supporting Information), this new green alga is sister to *Ignatius tetrasporus* and/or *Pseudocharacium americanum* within the Ignatius clade. The 18S rRNA-based phylogenetic tree strongly supports a sister relationship of this new green alga with the Ignatius clade but the divergence between this new green alga and the Ignatius clade is large.

Results of identity and coverage of nucleotide sequences from this new green alga, *Ignatius tetrasporus* UTEX2012 and/or *Pseudocharacium americanum* UTEX2112 are shown in Table 1. The coverage values of 18S rRNA or ITS2 or *rbc*L from this new green alga are more than 93% when compared with corresponding nucleotide sequences from *I. tetrasporus* UTEX2012 or *P. americanum* UTEX2112. The identity values of 18S rRNA, ITS2, or *rbc*L from this green alga are less than 89% when compared with corresponding nucleotide sequences from *I. tetrasporus* UTEX:2012 or *P. americanum* UTEX2112. It is strange to note that the identity of 18S rRNA or *rbcL* of *I. tetrasporus* UTEX2012 is nearly identical when compared with corresponding nucleotide sequences from *P. americanum* UTEX2112.

Diagnosis. This green alga is spherical and unicellular, with uninucleate and multiple chloroplasts with a pyrenoid. The aplanosporangium of this

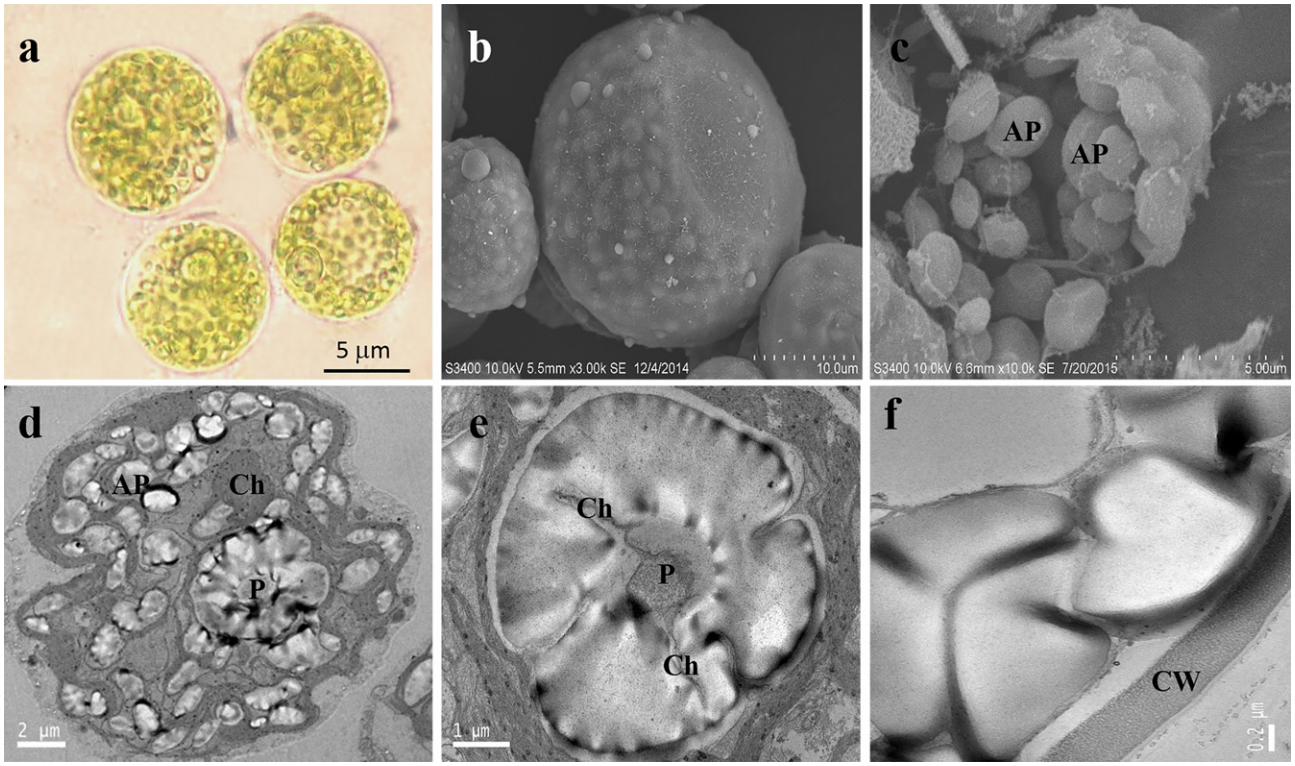

FIG. 2. Light, scanning electron, and transmission electron microscopic presentations of aplanosporangiums and autospore of *Symbiochlorum hainanensis*. (a) Light microscopy of aplanosporangiums. (b) SEM of aplanosporangiums. (c) SEM of fracturing aplanosporangiums and released autospore. (d) TEM of aplanosporangiums, scale = 2 μm. (e) TEM of pyrenoid in aplanosporangiums, scale = 1 μm. (f) TEM of aplanosporangiums, scale = 0.2 μm. P, pyrenoid; N, nucleus; V, vacuole; Ch, chloroplast; CW, cell wall; AP, autospore. [Color figure can be viewed at wileyonlinelibrary.com]

green alga contains many autospore. This green alga differs from *Ignatius tetrasporus* UTEX2012 or *Pseudocharacium americanum* UTEX2112 in Ignatius clade by the presence of spherical and unicellular vegetative cell with multiple chloroplasts and a distant phylogenetic relationship with Ignatius clade.

***Symbiochlorum hainanensis* S. Q. Gong & Z. Y. Li sp. nov.**

Description – Species description as for genus.

Diagnosis – Diagnosis information as for genus.

Holotypus – *Symbiochlorum hainanensis*, isolated from bleached *Porites lutea* living in the northern part of the South China Sea (109.470° E, 18.200° N). The holotype specimen of Ultra-thin sections of *S. hainanensis* is deposited in Shanghai Jiao Tong University, Marine Biotechnology Laboratory, China (MBLZ 2017001). The culture from which the holotype derives (MBLZ 2017001) has been accessioned by the China Center for Type Culture Collection (CCTCC M2018096).

Isotypus – Isotype specimens are also deposited in Shanghai Jiao Tong University, Marine Biotechnology Laboratory, China (MBLZ 2017002).

Etymology – The genus name of *Symbiochlorum* is a compound word, *Symbio* for association of algae with coral hosts, *chlo* is from Chlorophyta as this genus belong to Chlorophyta, and *rum* is used as affix. The species name of *hainanensis* is also a compound word, *hainan* refers to the sampling location, as *S. hainanensis* is isolated from bleached *Porites lutea* living in the northern part of the South China Sea (109.470°E, 18.200°N), near to the southern region of Hainan Island, China, and *ensis* is used as affix.

DISCUSSION

The Ulvophyceae includes mostly macroscopic, multicellular, or siphonous species from marine habitats but also certain microscopic unicellular or multicellular species from freshwater and terrestrial environments (Cocquyt et al. 2010, Darienko and Proschold 2017). Traditionally, species identification and their affiliation to the Ulvophyceae are often based on morphological traits of cells. The typical characteristics of the Ulvophyceae include cytomorphological types (e.g., a single nucleus and chloroplast or multicellular bodies or siphonocladous or siphonous morphological types), ultrastructural features (e.g., the orientation of the basal bodies in the flagellated cells), and sexual reproduction ("Codiolum"-stage; Cocquyt et al. 2010, Škaloud et al. 2013, Fang et al. 2017). However, for many algal species, the flagellated cells and sexual reproduction remain unknown or cannot be easy

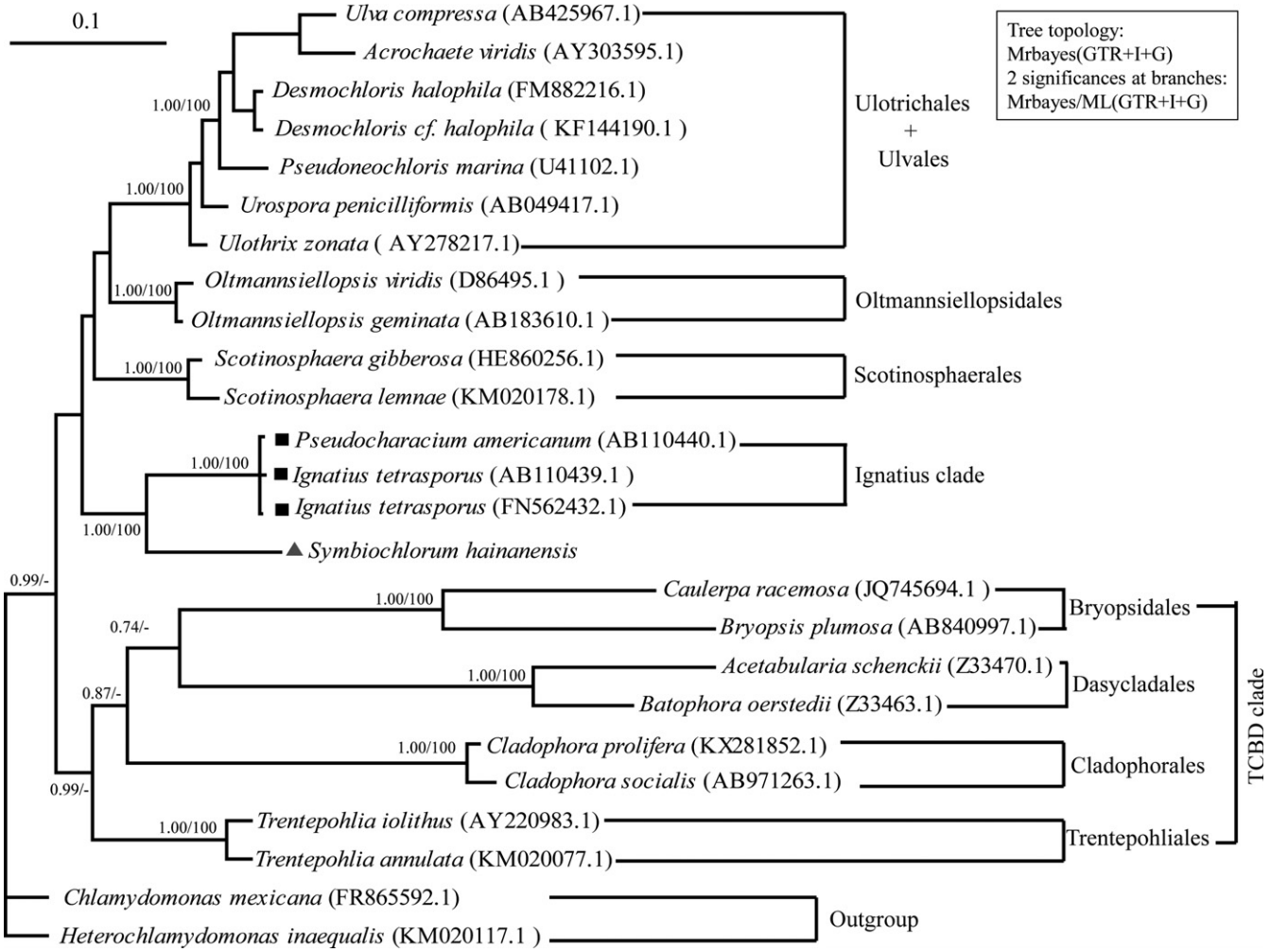

FIG. 3. Phylogeny of the Ulvophyceae based on complete 18S rRNA sequences (1,709 aligned nucleotide characters) from eight orders. The triangle indicates the position of *Symbiochlorum hainanensis*. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1. Summary of the identity and coverage values of nucleotide sequences. Including 18S rRNA, ITS2, and *rbc*L nucleotide sequences of *Symbiochlorum hainanensis*, *Ignatius tetrasporus* UTEX2012, and *Pseudocharacium americanum* UTEX2112.

	Symbiochlorum hainanensis					
	Identity (%)			Coverage (%)		
	18S	ITS2	*rbc*L	18S	ITS2	*rbc*L
I. tetrasporus UTEX2012	89 (99*)	61 (-)	73 (100*)	99 (99*)	93 (-)	99 (100*)
P. americanum UTEX2112	88 (99*)	–	73 (100*)	100 (99*)	–	99 (100*)

*Representing the identity and coverage values of nucleotide sequences of *I. tetrasporus* UTEX2012 compared with *P. americanum* UTEX2112.

observed (Cocquyt et al. 2010). Therefore, the molecular phylogeny of nucleotide sequences (18S rRNA or ITS2 and/or ITS, or *rbcL*) is used for most of the recent investigations of Ulvophyceae (Darienko and Proschold 2017). At present, five clades of green algae, including at least eight orders within the class Ulvophyceae, have been classified based on cytomorphological and phylogenetic analyses (Cocquyt et al. 2010, Škaloud et al. 2013, 2018, Fang et al. 2017).

In our study, a new green alga is isolated from bleached coral of *Porites lutea* living in the South China Sea. Based on light/scanning electron/transmission electron microscopy, and molecular

phylogenetic analyses using multiple nucleotide sequences, this new green alga is identified as *Symbiochlorum hainanensis* gen. et sp. nov., a sister lineage to the Ignatius clade, Ulvophyceae, Chlorophyta. *Symbiochlorum hainanensis* is unicellular marine alga and has uninucleate vegetative cells and multiple chloroplasts with a pyrenoid. While *Ignatius tetrasporus* UTEX 2012 (Bold and MacEntee 1974) and *Pseudocharacium americanum* UTEX 2112 (Lee and Bold 1974) belonging to the Ignatius clade were isolated from a soil sample of a white pine forest (PA, USA, North America) and the surface of thalli of an *Oedogonium* sp. in a river (America), respectively, they commonly have multinucleate and a single chloroplast with pyrenoid. In addition, *S. hainanensis* can form a cell wall-covered aplanosporangium. It reproduces by releasing many autospore by fracture of the cell wall of aplanosporangium. However, these morphological characteristics have not been reported in *I. tetrasporus* UTEX 2012 and *P. americanum* UTEX 2112. It has been reported that *I. tetrasporus* UTEX 2012 and *P. americanum* UTEX 2112 produce zoospores, and *I. tetrasporus* UTEX 2012 can form irregular tetrad aggregations composed of two to eight daughter cells within a mother cell wall (Bold and MacEntee 1974). These features suggest that *S. hainanensis* differs substantially from the two genera *Ignatius* (Bold and MacEntee 1974) and *Pseudocharacium* (Lee and Bold 1974).

Most importantly, the present phylogenetic analyses based on multiple nucleotide sequences reveal that *Symbiochlorum hainanensis* forms a sister branch to *Ignatius tetrasporus* UTEX 2012 and/or *Pseudocharacium americanum* UTEX 2112 within Ignatius clade, and the values of identity of all nucleotide sequences (18S rRNA or ITS2 or *rbc*L) of *S. hainanensis* are less than 89% when compared with those of *I. tetrasporus* UTEX 2012 and/or *P. americanum* UTEX 2112. All these results indicate that the phylogenetic divergence (Fig. 3) and nucleotide sequences difference (Table 1) between the *S. hainanensis* and the Ignatius clade are large, supporting that *S. hainanensis* is a new sister lineage to the Ignatius clade. Most recently, the Ignatius clade has been formally described as a new family Ignatiaceae in the order, Ignatiales (Škaloud et al. 2018). Although the large phylogenetic divergence and nucleotide sequences difference between *Symbiochlorum* and the Ignatius clade (Fig. 3) imply that *Symbiochlorum* would also be a new family or even new order, more comparative species or data are still needed.

Consistent with Watanabe and Nakayama (2007), our present results support the fact that *Ignatius tetrasporus* UTEX 2012 and *Pseudocharacium americanum* UTEX 2112 are grouped together and form a single clade within the Ulvophyceae. However, it is strange to note that the identity of 18S rRNA or *rbc*L of *I. tetrasporus* UTEX2012 is nearly identical when compared with corresponding nucleotide sequences from *Pseudocharacium americanum* UTEX2112. Therefore, we suspect earlier studies to be erroneous in the identification of *Ignatius tetrasporus* UTEX 2012 and *P. americanum* UTEX 2112 as different genera and species (Bold and MacEntee 1974, Lee and Bold 1974).

In summary, a new green alga isolated from bleached coral is described as *Symbiochlorum hainanensis* gen. et sp. nov., which forms a new sister lineage to the Ignatius clade, Ulvophyceae, Chlorophyta. Our future study will focus on a more extensive set of experiments (i.e., to isolate new algal species or strains belonging to *Symbiochlorum*, and cultivation *S. hainanensis* as well as *I. tetrasporus* UTEX 2012 and *P. americanum* UTEX 2112 under different conditions) for placing the *Symbiochlorum* in family or even order level. Meanwhile, a more extensive set of culture experiments will be performed for understanding the alga's life on corals, as *S. hainanensis* was isolated from bleached corals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Phylogeny of the Chlorophyta based on complete 18S rRNA sequences (1,709 aligned nucleotide characters) from nine typical classes. The red triangle indicates the position of *Symbiochlorum hainanensis*. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values.

Figure S2. Phylogeny of the Ulvophyceae based on complete ITS2 sequences (307 aligned nucleotide characters, including 5.8S rRNA and 28S rRNA partial sequences) from seven orders. The red triangle indicates the position of *Symbiochlorum hainanensis*. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values.

Figure S3. Phylogeny of the Ulvophyceae based on partial *rbc*L sequences (555 aligned nucleotide characters) from eight orders. The red triangle indicates the position of *Symbiochlorum hainanensis*. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values.