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The morphological and phylogenetic characterization for the dinoflagellate *Margalefidinium fulvescens* (=*Cochlodinium fulvescens*) isolated from the Jiaozhou Bay, China

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Abstract

The dinoflagellate genus *Margalefidinium* has been split from *Cochlodinium* as a new genus recently and *Margalefidinium fulvescens* is one of the five *Margalefidinium* species. *Margalefidinium fulvescens* is toxic and has been reported from the coastal waters of USA, Canada, Mexico, China, Japan, Indonesia, Korea, Pakistan and Spain. Here we provide the morphological and phylogenetic characterization for an isolate of it from the Jiaozhou Bay, Qingdao, China. Our results showed that the vegetative cells were subspherical to ellipsoidal, 34–60 μm in length, and 19–41 μm in width. Both single cell forms and colonies in chains of 2, 4, or 8 cells were observed in cultures, but chain forms with 2 or 4 cells were observed more often in the field samples. The cingulum was rather deep, encircling the cell approximately twice, but the sulcus was rather narrow, surrounding the cell about one turn. The nucleus was spherical and located at the central epicone. The chloroplasts were granular, brownish, and scattered peripherally. An orange pigmented body also appeared in the epicone. The apical groove appeared vase-like as previously described. Under epi-fluorescence microscopy, a pumpkin-like structure was clearly observed, in which cells were embedded. Cells were observed to exit from the structure, which led us to a hypothesis that the structure may provide cells a shelter to avoid predation or to respond to other stresses. The phylogenetic analyses based on partial LSU rDNA sequences indicated that *M. fulvescens* from the Jiaozhou Bay was grouped with *M. fulvescens* populations from other origins and closely related to the clade of *M. polykrikoides*. Our morphological observations and phylogenetic analyses together confirmed the presence of *M. fulvescens* in China and our monitoring has also observed the species dominant in the dinoflagellate community of the Jiaozhou Bay in the early autumn of 2015, which alerted us to continually monitor this bloom-forming species in the region.

Key words: *Margalefidinium fulvescens*, morphology, phylogeny, pumpkin-like structure, Jiaozhou Bay, China

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1 Introduction

The genus *Cochlodinium* F. Schütt belongs to the order Gymnodiniales, which was established by Schütt in 1896, including species with a distinctive spiral-shaped cingulum that makes more than 1.5 turns around the cell (Schütt, 1896; Kofoid and Swezy, 1921). There have been about 35 species described in this genus (Guiry and Guiry, 2011; Gómez, 2012; Kudela and Gobler, 2012). As more laboratory cultures of *Cochlodinium* species were more carefully investigated, especially for those photosynthetic species, their fine morphological features (apical groove, the relative position of cingulum and sulcus, chloroplast shape, eyespot, etc. observed both by light and electron microscopy) and molecular sequences have indicated that these species are not mono-phylogenetic, which should be split into several genera (Iwataki et al., 2007, 2015; Reñé et al., 2013, 2015). In a recent study, the genus of *Cochlodinium* was emended, in which the type species of this genus, *Cochlodinium strangulatum*, was re-investigated from the type locality (the Atlantic Ocean) and com-

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pared with *C. polykrikoides*, *C. fulvescens*, and other dinoflagellates regarding their morphology and molecular identity in rDNA genes (Gómez et al., 2017). Based on the morphology of apical groove, cingulum turns, perinuclear capsule, forming chained colony, and molecular phylogeny, these described *Cochlodinium* species have been classified into two genera: *Cochlodinium* Schütt emend. F. Gómez, Richlen and D. M. Anderson, and *Margalefidinium* F. Gómez, Richlen and D. M. Anderson. As a consequence, *C. polykrikoides* and *C. fulvescens* were placed in the new genus *Margalefidinium*, which includes other three species (Gómez et al., 2017).

Margalefidinium polykrikoides (Margalef) Gómez, Richlen and Anderson, gen. & comb. nov., has been intensively studied since it produces ichthyotoxins and formed catastrophic blooms causing massive fish mortalities from coastal waters of the Caribbean Sea, West Pacific Ocean, West Atlantic Ocean, Indian Ocean, Mediterranean Sea, and the Arabian Gulf (Richlen et al., 2010; Kudela and Gobler, 2012; Tang and Gobler, 2012; Iwataki et al., 2015; Harun et al., 2015). It is noteworthy that a bloom of *M. polykrikoides* occurred in the Arabian Gulf and Gulf of Oman in 2008–2009 lasted for more than ten months and caused thousands of tons of fish mortality, damage to coral reefs, negative impacts to coastal tourism, and the forced closure of desalination plants in the region (Richlen et al., 2010; Al-Azri et al., 2014). Therefore, *Margalefidinium* has been one of the most important and most intensively investigated HAB groups.

Margalefidinium fulvescens (Iwataki, Kawami and Matsuoka) Gómez, Richlen and Anderson, described as *C. fulvescens* in 2007 from Asian coasts (Iwataki et al., 2007), is also well known as a HABs-causing species since blooms of which caused substantial mortality to farmed salmon in the west coast of Canada and USA (Whyte et al., 2001; Kudela and Gobler, 2012). *Margalefidinium fulvescens* and *M. polykrikoides* are not easy to be distinguished morphologically under light microscopy since they both form chain colonies and have shallow sulcus without harboring the longitudinal flagellum (Iwataki et al., 2007, 2015; Matsuoka et al., 2008). They, however, differ in chloroplast shape, eyespot, morphology of apical groove, and the position of sulcus (Iwataki et al., 2007, 2008; Matsuoka et al., 2008). Because of the similar morphological features shared by different *Margalefidinium* species or morphological deformation during preservation, *M. fulvescens* was identified as *Margalefidinium* sp. only, or misidentified as *M. polykrikoides*, *M. catenatum*, *Gymnodinium impudicum*, and even *Alexandrium catenella* in previous studies (Whyte et al., 2001; Cho and Costas, 2004; Curtiss et al., 2008; Morquecho-Escamilla and Alonso-Rodríguez, 2008; Howard et al., 2012). The presence of *M. fulvescens*, however, has been confirmed in the west coasts of USA and Canada, Mexico, Japan, Indonesia, Korea, Pakistan, and Spain (Iwataki et al., 2007, 2008; Munir et al., 2012; Gárate-Lizárraga, 2014; Reñé et al., 2015; Thangaraj et al., 2017), while a report of its presence in China was based on light microscopy morphology only (Pan et al., 2012). Although bloom events of *M. fulvescens* were only recorded from the west coasts of Canada and USA, this organism probably possesses ichthyotoxic properties similar to *M. polykrikoides* (Whyte et al., 2001; Curtiss et al., 2008; Iwataki et al., 2008; Howard et al., 2012). Therefore, it is important to investigate its geographic distribution, possible toxicity to aquatic animals, and allelopathy to other phytoplankton species.

During a routine monitoring of HABs in the Jiaozhou Bay, China from May to October 2015, we found both *M. polykrikoides* and *M. fulvescens* presented in the bay and the adjacent coastal waters from August to October, with the total cell density of *M. fulvescens* and *M. polykrikoides* reaching 68 000 cells/L. We then established clonal cultures of *M. fulvescens* and here we report our observation on the morphology via light and scanning electron microscopy and phylogenetic analyses based on LSU rDNA sequences for the isolate.

2 Materials and methods

2.1 *Sample collection and establishment of cultures*

The clonal culture of *M. fulvescens* (strain No. MFJZB1) was established by single cell micropipetting from samples collected from the Jiaozhou Bay, Qingdao, China on August 11, 2015. The culture was grown in natural seawater with a salinity of 33 enriched with f/2-Si medium (Guillard, 1975) and 10^{-8} mol/L selenium (final concentration) under (21±1) °C, an irradiance of ~100 μmol quanta m^{-2} s^{-1}, and a photoperiod of 12 h:12 h (light:dark) supplied by white fluorescent lights. An antibiotic solution (a mixture of 10 000 IU penicillin and 10 000 μg/mL streptomycin, Solarbio, Beijing, China) was added into the medium immediately before inoculation (final concentration 2%) to inhibit bacterial growth.

2.2 *Light microscopy (LM)*

Vegetative cells were observed under an inverted microscope (IX73, Olympus, Japan) and upright microscope (BX53, Olympus, Japan) using normal bright field and photographed with a digital camera (model Olympus DP80). Cells in mid-exponential growth phase were fixed with Lugol's solution at a final concentration of 5%, and the cell sizes of 50 cells were measured at 200× magnification. The sizes of a pumpkin-like structure and holes on it (see Results Section) were measured for 20 and 10 individual structures. For epi-fluorescence microscopy, live, healthy cells of 1 mL were stained with SYBR Green (Solarbio, Beijing, China), viewed and photographed with the abovementioned digital camera for chlorophyll-induced red autofluorescence and green fluorescence of nucleus caused by SYBR Green staining.

2.3 *Scanning electron microscopy (SEM)*

Cells at exponential growth phase were harvested and fixed with OsO_4 (2% final concentration) dissolved in f/2-Si culture medium for 40–50 min, gently filtered onto a 11 μm Millipore nylon membrane, dehydrated in an acetone series (10%, 30%, 50%, 70%, 90%, and 3 times in 100%, 15 min for each step), critical point-dried with liquid CO_2 (automated critical point dryer, EM CPD 300, Leica, Austria), sputter-coated with gold (Sputter/Carbon Thread, EM ACE200, Leica, Austria), and observed with an S-3400N SEM (Hitachi, Japan).

2.4 *DNA extraction, PCR amplification and LSU rDNA sequencing*

Total DNA of *M. fulvescens* was extracted from 10 mL of exponentially growing culture using a plant DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's protocol. Approximately 1 400 bp of LSU rDNA was amplified using the primers D1R (Scholin et al., 1994) and 28-1483R (Daugbjerg et al., 2000). The PCR reactions were performed in a 25 μL system, containing 9.5 μL ddH_2O, 12.5 μL 2×Taq PCR MasterMix, 1 μL of each PCR primer (10 mmol/L), and 1 μL DNA template. The amplification was performed with an initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min, and a final elongation step of 10 min at 72°C. The PCR products were checked in a 1% agarose gel containing ethidium bromide and visualized with ultraviolet light. The targeted bands were purified by agarose gel DNA fragment recovery kit (GENEray Bio-

technology, Shanghai, China), ligated with pMD-18T cloning vector (TaKaRa, Tokyo, Japan), and then sequenced (Sangon, Shanghai, China). The obtained sequence was deposited in GenBank with an accession number MF351924.

2.5 *Sequence alignment and phylogenetic analyses*

For comparison and phylogenetic analyses, LSU rDNA sequences of *M. fulvescens* and other 15 close taxa were used for phylogenetic analyses (their accession numbers are shown after each taxon in the phylogenetic tree). A sequence of *Pyrodinium bahamense* (accession No. AY154959) was used as the outgroup. The sequences were aligned using the MAFFTv.7 with the default settings (Katoh et al., 2002) (http://mafft.cbrc.jp/alignment/server/) and modified manually in BioEdit (v7.2.5) (Hall, 1999). The final alignment of LSU rDNA sequences included 670 positions after alignment relative to our sequence. TIM3+G was the best-fit model selected by jModeltest 2.2 under the AICc criterion (Posada, 2008). Maximum Likelihood (ML) analysis was performed using PhyML (Guindon et al., 2010), with the TIM3+G model and 1 000 bootstrap replicates. The Bayesian Inference (BI) analysis was conducted with MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003). Four independent Markov chain Monte Carlo simulations were run simultaneously for 10 000 000 generations and trees were sampled every 1 000 generations. The first 25% trees were discarded as burn-in. The convergence was judged based on the average standard deviation of split frequencies (all less than 0.01). The remaining trees were used to generate a consensus tree and calculate the posterior probabilities of all branches using a majority-rule consensus approach. FigTree (v1.4.3) were used to view and edit trees for publication.

3 Results

The vegetative cells of *M. fulvescens* were subspherical to ellipsoidal, and 34–60 μm (mean=46 μm, *n*=50) in length, and 19–41 μm (mean=29 μm, *n*=50) in width (Figs 1 and 2). Single cell, two-, four-, or eight-cell chains were observed in the culture (Fig. 1), while two- or four-cell chains were observed more often in the field samples from the Jiaozhou Bay, China. Both the cingulum and sulcus started from the same position (Figs 1 and 2). The cingulum was rather deep, and surrounded the cell approximately twice, harboring the transverse flagellum, while the sulcus was narrow, encircling the cell about once, and never held the longitudinal flagellum in this structure (Figs 1 and 2). The nucleus was spherical and located in the center of the epicone (Figs 1b and d). Chloroplasts were usually granular and brownish and scattered peripherally (Figs 1b, c and e). An orange pigmented body also appeared in the epicone (Fig. 1c). The apical groove appeared like a vase (Fig. 2), as termed in Iwataki et al. (2015).

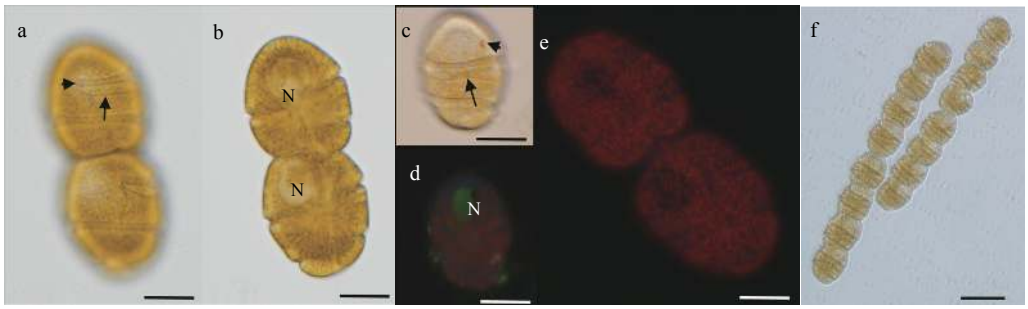

Fig. 1. Light microscopic micrographs of *Margalefidinium fulvescens*. a. Ventral view of a two-cell chain showing the starting position of the cingulum and sulcus (arrowhead) and the narrow sulcus (arrow); b. deep focus of the same cells of Fig. 1a, a spherical nucleus (N) located in the central epicone; c. dorsal view showing an orange pigmented body (arrowhead) located in the epicone, and sulcus (arrow) running in the intermediate position of the cingulum; d. SYBR Green-stained cell in dorsal view, showing a nucleus (N); e. epifluorescence micrograph showing granular chloroplasts positioned peripherally; and f. a chain colony consisting of eight cells, surface view. Scale bars for a, b, c, d, e are 20 μm, and for f is 50 μm.

In the culture, single cell, two- or four-cell chains were sometimes found to be embedded in a sac or shell with a pumpkin-like morphology, as clearly observed under epi-fluorescence LM (Fig. 3). The pumpkin-like structure was spherical, with a diameter of 89–119 μm (mean=102 μm, *n*=20) and a thickness 36–63 μm (mean=51 μm, *n*=10). A hole was observed on the pumpkin-like structure, with a diameter of 9–22 μm (mean=14 μm, *n*=20). The structure contained two shields combining together, and there was one hole on each side of the structure, but the sizes of the two holes were different (Fig. 3). On each side of the structure, there were many equidistant furrows covering on it, and the radiating furrow started from the central hole and ended at the other side of the sphere (Figs 3b and c). Vegetative cells were observed to swim freely within the structure (Fig. 3a) and occasionally single or 2-cell, 4-cell chain cell(s) were observed to escape from the shell: firstly, the epicone part shrunk and squeezed out from the hole, and recovered to its normal morphology, then the other part of the cell(s) followed the same route to escape from the structure, and the cell(s) moved freely as usual in the medium (Fig. 4).

A partial LSU rDNA sequence (1 435 bp, GenBank accession No. MF351924) of *M. fulvescens* was obtained from the clonal culture of *M. fulvescens*. The sequence was blasted with BLASTn in http://blast.ncbi.nlm.nih.gov/Blast.cgi and found to be 98% (1 395 bp/1 417 bp) and 99% (1 434 bp/1 435 bp) identical to two entities of this species collected from California, USA (accession No. AB295051) and the Tachibana Bay, Japan (accession No. AB288382), respectively.

The analyses of ML and BI based on LSU rDNA sequences generated similar phylogenetic trees (Fig. 5). All entities of *M. fulvescens* from China, Japan, Canada, USA, and Spain grouped together with a strong support, and they together separated from the sister group of *M. polykrikoides* (Fig. 5). In the clade of *M. fulvescens*, however, the sequence from Canada distinguished from the others, because this sequence (906 bp) has four inserts containing 2, 3, 6 and 21 bases that were not present in all other sequences including the one from California, USA. The entity from Spain is also somehow separated from those from China, Japan

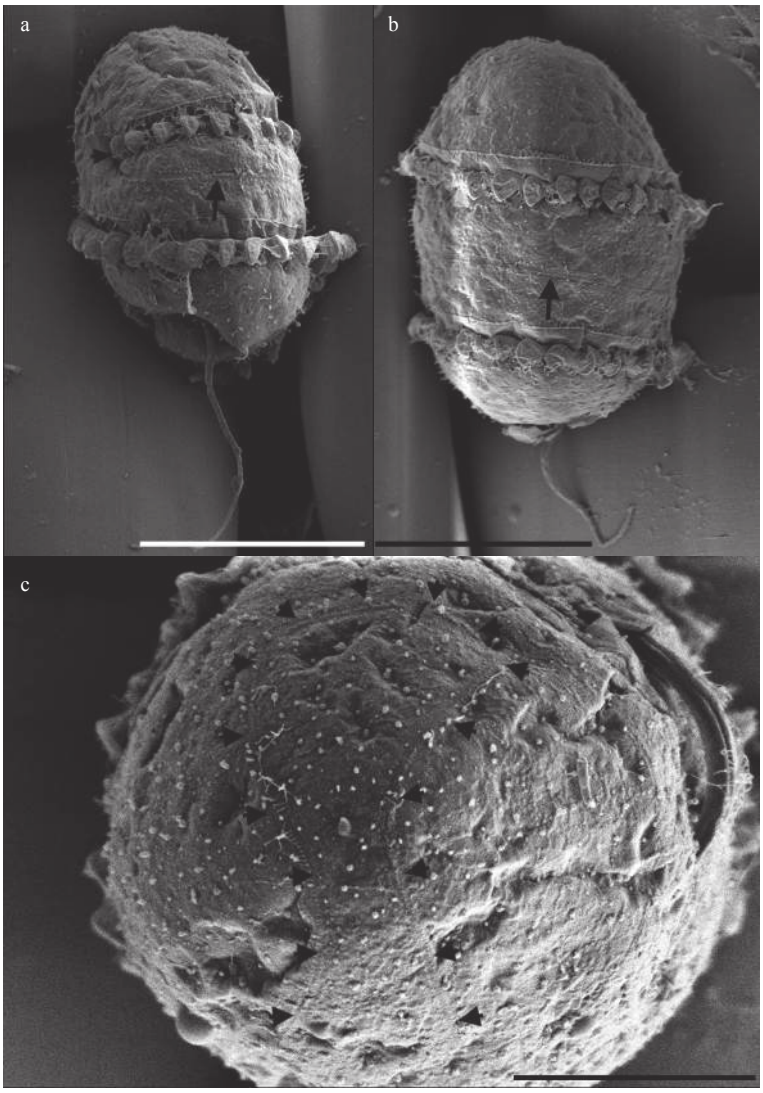

Fig. 2. Scanning electron microscopy of *Margalefidinium fulvescens*. a. Ventral view showing the starting position of the cingulum and sulcus (arrowhead) and the narrow sulcus (arrow), and transverse (tf) and longitudinal flagella (lf); b. dorsal view showing the sulcus (arrow) running in the intermediate position of the cingulum; c. apical view showing the apical groove (multiple arrowheads). Scale bars: 20 μm (a and b) and 10 μm (c).

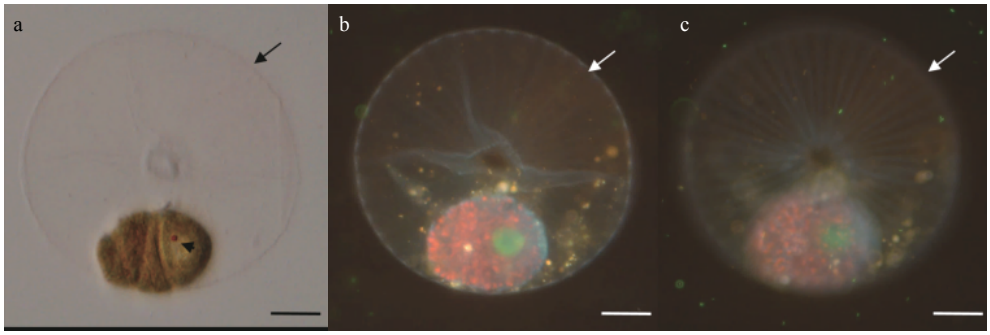

Fig. 3. Light micrographs of *Margalefidinium fulvescens* cells enclosed in a pumpkin-like structure (arrow showing the edge of pumpkin-like structure). a. Dorsal view showing an orange pigmented body (arrowhead); b and c. SYBR Green-stained cell under different focus. Scale bars are 20 μm.

and USA, which was obviously due to its relatively short length (635 bp) as there was no significant difference in its sequence as aligned to those from China, Japan and USA.

4 Discussion

In the past 30 years, species of the toxic dinoflagellate genus *Margalefidinium* (as *Cochlodinium*) have been observed to rap-

Fig. 4. Light micrographs showing two-cell (a) and four-cell chains (b and c) of *Margalefidinium fulvescens* exiting from the pumpkin-like structure. Scale bars are 20 μm.

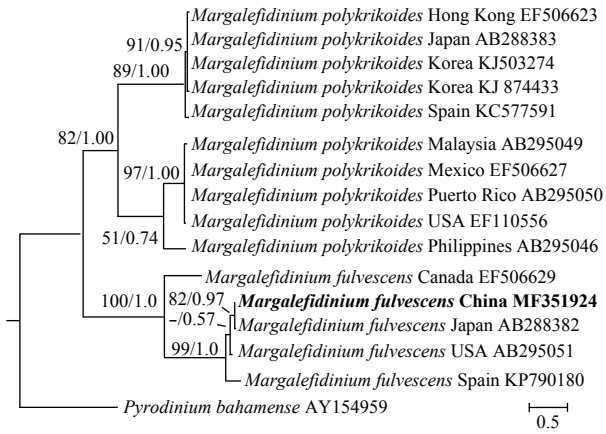

Fig. 5. Molecular phylogeny of Chinese strain of *Margalefidinium fulvescens* inferred from partial large subunit rDNA sequences based on maximum likelihood (ML) and Bayesian inference (BI). Only bootstrap values >50 and BPP >0.5 are shown. *Pyrodinium bahamense* was used as an outgroup. Nodal support for branches in the ML and BI trees is marked in order (ML/BI). All branches are drawn to scale.

idly expand geographic distribution and form numerous catastrophic blooms and therefore have been considered to be one of the few most important HAB-forming species (Curtiss et al., 2008; Matsuoka et al., 2008; Richlen et al., 2010; Kudela and Gobler, 2012). In China, the first *M. polykrikoides* bloom was reported in 1984 from the Tolo Harbor, Hong Kong and then several blooming events were recorded in the southern and southeastern waters of Hong Kong in 1998 and 2011 with no fish kill reported (Law and Lee, 2013). In 1990, a bloom of *Cochlodinium* sp. occurred in the coastal water of Fujian (Du et al., 1993), but the species identity has never been confirmed. In 2006, several *M. polykrikoides* blooms were recorded in the Zhujiang (Pearl River) Estuary, Guangdong Province (Guangdong Ocean and Fishery Bureau, 2006). In 2010 and 2011, blooms of *M. polykrikoides* were also reported in the coastal waters of Zhejiang Province (Yu et al., 2011; Wang et al., 2014). In 2012, a small scale of *M. polykrikoides* bloom was reported in the coastal water of Qingdao, Shandong Province (North China Sea Branch of State Oceanic Administrate, People's Republic of China, 2012). In 2014, *M. polykrikoides* was reported to be the dominant species in the phytoplankton community in the Haizhou Bay, Jiangsu Province (Bulletin of Aquaculture Environment Quality in HABs Monitoring Regions, National Marine Environmental Monitoring Center of SOA, 2014). From 2014 to 2016, *M. polykrikoides* consecutively bloomed in the same coastal area of Tianjin (North China Sea Branch of State Oceanic Administrate, People's Republic of China, 2014, 2015, 2016). These blooms of *M. polykrikoides*, although no serious fish kill was reported, indicate an obvious geographic expansion of this species along the coast of China and as a consequence, have attracted a great attention from the research community of HABs and local governments.

Margalefidinium fulvescens was described as a new species in 2007 and recently moved from *Cochlodinium* to *Margalefidinium* (Iwataki et al., 2007; Gómez et al., 2017). Although there were many reports of *M. polykrikoides* or *Margalefidinium* sp. in Chinese coastal waters, unambiguous morphological observation is scarce, which caused uncertainty in those identifications. As known from some previous reports and our recent observations, *M. fulvescens* and *M. polykrikoides* may often coexist in some coastal waters (Gárate-Lizárraga, 2014; Thangaraj et al., 2017; this study). Also, *M. fulvescens* is not easy to be distinguished from *M. polykrikoides*, *M. catenatum* (possibly conspecific to *M. polykrikoides*), *G. impudicum*, or even *A. catenella* under low magnifications of microscopy, especially for samples fixed with preservatives that may deform fragile unarmored dinoflagellates (Whyte et al., 2001; Cho and Costas, 2004; Curtiss et al., 2008; Morquecho-Escamilla and Alonso-Rodríguez, 2008; Howard et al., 2012; Gómez et al., 2017). In addition, since *M. fulvescens* was described in 2007, all identifications of *C. polykrikoides* based on light microscopic observations only prior to 2007 are thus questionable if the major features distinguishing *M. polykrikoides* from *M. fulvescens* were not clearly shown or stated (the morphology of apical groove and the relative positions of cingulum and sulcus), or no molecular data were provided. Therefore, we believe it is important to provide a detailed morphological characterization and molecular phylogeny for *M. fulvescens* in the coastal waters of China, and to compare it with other isolates from different geographic locations.

From August to October of 2015, during our monitoring of the phytoplankton composition and dynamics in the Jiaozhou Bay, China, we noticed that *M. polykrikoides* and *M. fulvescens* coexisted and their total cell density ranged from 2 000 to 68 000 cells/L, which might be considered as a slight bloom, with no apparent deleterious effect. We then established clonal cultures of *M. fulvescens*. All the important morphological features including cell size, cell numbers in colonies, turns of cingulum, shape and location of chloroplasts, and particularly the position of the narrow sulcus and shape of apical groove (aka acrobase) are consistent to the original descriptions of the species (Iwataki et al., 2007, 2015), which was further confirmed by our molecular phylogenetic analyses. Therefore, the present work presents the first un-

ambiguous identification of *M. fulvescens* from Chinese coastal waters.

For the sake of accurately identifying *M. fulvescens* and *M. polykrikoides* from the same sample, it is worthy of notice that while both species form two-, four-, or eight-cell chains, *M. fulvescens* does not form 16-cell chain (Tomas and Smayda, 2008; this study). Other two important diagnostic features discernible under light microscopy that distinguish *M. fulvescens* from *M. polykrikoides* are the relative position of sulcus and cingulum and shape and location of chloroplasts (Figs 1 and 2; Iwataki et al., 2007, 2015; Matsuoka et al., 2008). However, the difference in the apical groove between these two species is discernible only under SEM. The pumpkin-like structure was observed in our clonal culture but not observed from field samples and other reports. Since it was observed that cells could leave this structure freely, we assume this structure may possibly provide cells a protective mechanism to avoid grazing or respond to stress. We are uncertain, however, about whether or not the structure is of any taxonomic and other ecological significance.

In the phylogenetic trees based on the partial LSU rRNA gene, we noticed that all entities of *M. fulvescens* from China, Japan, USA and Spain seemed to be genetically distant from that from Canada, suggesting an inter-populational diversity, which certainly deserves a further in-depth investigation.

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