Algal Research 22 (2017) 14–30

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Morphology, molecular phylogeny and okadaic acid production of epibenthic *Prorocentrum* (Dinophyceae) species from the northern South China Sea

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ARTICLE INFO

Article history:
Received 21 June 2016
Received in revised form 26 October 2016
Accepted 27 November 2016
Available online xxxx

Keywords:
Diarrheic shellfish poisoning
Okadaic acid
Prorocentrum emarginatum
Prorocentrum hoffmannianum
Prorocentrum maculosum
Prorocentrum panamense

ABSTRACT

Around 30 epibenthic *Prorocentrum* species have been described, but information about their biogeography is limited. Some species are able to produce okadaic acid (OA) and its derivatives, which are responsible for diarrheic shellfish poisoning (DSP). In the present study, we examined the diversity of epibenthic *Prorocentrum* in the northern South China Sea by isolating single cells from sand, coral, and macroalgal samples collected from 2012 to 2015. Their morphology was examined using light microscopy and scanning electron microscopy. Among 47 *Prorocentrum* strains, seven morphospecies were identified as *P. lima*, *P. rhathymum*, *P. concavum*, *P.* cf. *emarginatum*, *P. fukuyoi*, *P.* cf. *maculosum* and *P. panamense*. The latter five species have not been previously reported in Chinese waters, and this is the first record of *P. panamense* outside its type locality. Partial large subunit (LSU) ribosomal DNA and internal transcribed spacer region sequences were obtained and molecular phylogenetic analysis was carried out using maximum likelihood and Bayesian inference. Chinese *P.* cf. *maculosum* strains share 99.5% similarity of LSU sequences with the strain from Cuba (close to the type locality), but Chinese *P. lima* strains share only 96.7% similarity of LSU sequences with the strain from the type locality. *P.* cf. *emarginatum* differs from *P. fukuyoi* mainly in the presence/absence of marginal pores and they form a well-resolved clade together with *P. sculptile*. OA was detected in all Chinese strains of *P. lima* and *P.* cf. *maculosum* based on liquid chromatography-mass spectrometry analysis, but dinophysistoxin was produced only by two *P. lima* strains. Chinese strains of *P. concavum*, *P. rhathymum*, and *P. panamense* do not produce detectable level of OA. Our results support the wide distribution of epibenthic *Prorocentrum* species and highlight the potential risk of DSP in the northern South China Sea.

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1. Introduction

Among dinoflagellates, the prorocentroid tabulation is characterized by the lack of a girdle and sulcus and by the presence of an apical periflagellar area where two flagella emerge. The order Prorocentrales was established to incorporate prorocentroid species, and it contains a single family (Prorocentraceae) and three genera (*Prorocentrum* Ehrenberg, *Haplodinium* Kleb, and *Mesoporus* Lillick) [23]. *Haplodinium* might be a junior synonym of *Prorocentrum* [46], and the phylogenetic position of *Mesoporus* remains to be determined.

The genus *Prorocentrum* was erected with *P. micans* Ehrenberg as the type species [17]. Later, *Exuviaella* Cienkowski was established to include *E. marina* Cienkowski (= *P. lima* (Ehrenberg) F. Stein) [10]. These two genera differ only in the presence or absence of an apical spine, and Abe [1] proposed that they be merged. [16] formally made *Exuviaella* a junior synonym of *Prorocentrum* and reduced the number of *Prorocentrum* species from 64 to 21 (most of them are planktonic), as great infraspecific variations were observed.

The genus *Prorocentrum* currently contains approximately 80 species divided nearly equally among planktonic and epibenthic lifestyles. Epibenthic *Prorocentrum* species inhabit intertidal marine sediments, macroalgal surfaces, floating detritus and corals [21]. The presumable epibenthic species (e.g., *P. arabianum* Morton & Faust (= *P. concavum* Fukuyo) and *P. rhathymum* A. R. Loeblich III, Sherley & Schmidt) can also be recovered from the plankton [12,50].

The morphology of *Prorocentrum* is rather simple, consisting of two large plates (valves) and a periflagellar area with several platelets. The useful characters for differentiation at the species level include cell shape and size, thecal plate surface (ornamentation and pore patterns), intercalary band morphology, and the number and shape of platelets and relevant structure (e.g., spines, collars, and protrusions in the periflagellar area). Among these traits, the periflagellar structure is

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http://dx.doi.org/10.1016/j.algal.2016.11.020
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regarded as being conservative and significant for differentiating species [33]. Other features such as cell shape and the number and shape of thecal pores have been reported to be variable [19,57,74]. The details of the periflagellar area in some species (e.g., *P. fukuyoi* S. Murray & Y. Nagahama) are still not clear [33]. Despite their morphological synapomorphies, *Prorocentrum* appeared to be polyphyletic or palyphyletic based on either small subunit (SSU) or large subunit ribosomal DNA (LSU rDNA) sequences [25,53]. However, they appeared to be monophyletic once concatenated data, especially from mitochondrial sequences, were used [59,73].

In view of the high morphological similarity among *Prorocentrum* species, it is not surprising that some species have been described repeatedly. For instance, *P. arabianum*, *P. arenarium* Faust and *P. minimum* (Pavillard) Schiller are now considered to be synonyms of *P. concavum*, *P. lima*, and *P. cordatum* (Ostenfeld) Dodge, respectively [48,57,70]. *P. mexicanum* Osorio-Tafall and *P. rhathymum*, as well as *P. hoffmannianum* Faust and *P. belizeanum* Faust might be conspecific as well [12,30]. *P. maculosum* Faust was described from Twin Cays, Belize and differs from *P. lima* only in the shape of valve pores and the relative size of flagella and accessory pores [20]. *P. emarginatum* Fukuyo was described from Okinawa, Japan using light microscopy [24]. To date, detailed morphology and molecular sequence data for *P. emarginatum* from the type locality are not available. *P. fukuyoi* differs from *P. emarginatum* mainly in cell shape, but *P. fukuyoi* is genetically very close to *P. emarginatum* from Fiji [54]. Thus, detailed information about *P. maculosum* and *P. emarginatum* is needed to understand the species boundaries.

Most epibenthic *Prorocentrum* species have been described from tropical or subtropical areas, but some can inhabit temperate areas. *P. lima* is considered to be a cosmopolitan species [57], and some species, including *P. rhyathymum* and *P. concavum*, are known to have a wide distribution [3,4,24,43]. In contrast, other species are rarely reported. For instance, *P. panamense* Grzebyk, Sako & Berland and *P. maculosum* have been reported only from Central America [20,25].

The most interesting feature of *Prorocentrum* is that some species are able to produce okadaic acid (OA) and its related derivatives (dinophysistoxins, DTXs), which are responsible for diarrheic shellfish poisoning (DSP). Some *Prorocentrum* species can produce other toxins as well, such as prorocentin [45] and formosalides [44]. OA is a polyether derivative of 38-carbon fatty acid and was first isolated from two marine sponges [65]. It is a potent tumor promoter that is a powerful inhibitor of protein phosphatases-1 and -2A [31,66]. Human diarrheic poisonings have occurred due to consumption of clams and crabs contaminated with OA esters [68]. OA production has been reported in epibenthic *P. lima*, *P. rhathymum*, *P. hoffmannianum*, *P. maculosum*, *P. levis* M.A. Faust, Kibler, Vandersea, P.A. Tester & Litaker, and *P. belizeanum* and in one planktonic species *P. texanum* Henrichs, Steidinger, Scott & Campbell [5,22,29,49,51,52,76]. *P. lima* strains are always toxic, producing OA and its analogues in varying quantities [7,32,67,72]. *P. rhathymum* from Florida, USA and Malaysia can produce OA [5,8], but those from Okinawa, Japan [72] and Greece [3] do not produce detectable toxin based on bioassay and protein phosphatase 2A inhibition assay. To date, OA production by *P. maculosum* has been reported qualitatively only [76]. Thus, OA production in more epibenthic *Prorocentrum* strains needs to be examined to better understand its prevalence.

Ten epibenthic *Prorocentrum* species have been reported from East Malaysia [47], and four have been reported in Vietnam waters [39]. So far, only *P. lima* and *P. rhathymum* have been reported in Chinese waters [42,74], but a richer diversity of epibenthic *Prorocentrum* can be expected. OA was detected in shellfish harvested from the South China Sea [71], but OA production was confirmed in only one strain of *P. lima* from Hainan [41]. The goal of the present study was to fully understand the diversity and OA production of epibenthic *Prorocentrum* species in the northern South China Sea.

2. Materials and methods

2.1. Sample collection and treatment

Samples were collected from ten stations in the northern South China Sea from 2012 to 2015 (Fig. 1, for geographical coordinates see Table 1). The macroalgal, seagrass, dead coral reef and upper centimeter of sandy sediments were collected from the seabed by scuba divers, and deposited into bottles containing seawater collected at the same location. The samples were stirred vigorously to detach the epibenthic cells and the suspension settled in a composite settling chamber. The settled materials were subsequently sieved through 120 μm and 20 μm filters. The 20–120 μm fractions were rinsed with filtered seawater and transferred into a polycarbonate bottle. In the laboratory, single live cells were isolated from this material with a micropipette under an inverted microscope Eclipse TS100 (Nikon, Tokyo, Japan) into a 96-well culture plate containing 330 μL f/2-Si medium [27] or L1 medium [26]. The culture plate was incubated at 25 °C, 90 μmol photons m^{-2} s^{-1}, and a light:dark cycle of 12 h:12 h (hereafter, called "standard culture conditions"). The cultures were transferred to a 6-well culture plate later under the standard culture conditions.

2.2. Light microscopy (LM)

Live cells were examined and photographed using a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen, Germany) equipped with a Zeiss Axiocam HRc digital camera, or an Olympus BX 61 (Olympus, Tokyo, Japan) equipped with a QImaging Retiga 4000R digital camera (QImaging, Surrey, British Columbia, Canada). More than thirty cells were measured using Axiovision (4.8.2 version) or IMG Pro plus (6.0 version) software at ×400 magnification. To observe the shape and location of the nucleus, cells were stained with 1:100,000 Sybr Green (Sigma Aldrich, St. Louis, USA) for 1 min, and photographed under the Zeiss fluorescence microscope with a Zeiss-38 filter set (excitation BP 470/40, beam splitter FT 495, emission BP 525/50). Chloroplast autofluorescence microscopy was carried out on live cells using a Leica DM6000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a B/G/R filter cube (blue: emission filter BP420/30, dichromatic mirror 415, suppression filter BP465/20; green: BP495/15, 510, BP530/30; red: BP570/20, 590, BP640/40), and digitally photographed using a Leica DFC300 FX digital camera.

2.3. Scanning electron microscopy (SEM)

Mid-exponential batch cultures were concentrated by a Sorvall Biofuge Primo R (Thermo Scientific, Massachusetts, USA) at 1250*g* for 10 min at room temperature. The cell pellet was re-suspended in 60% ethanol for 1 h at 8 °C to strip off the mucilage. The cells were centrifuged again to remove the ethanol and the pellet was fixed at 8 °C for 3 h with 5% glutaraldehyde prepared with filtered seawater. Cell pellets were washed twice with filtered seawater and fixed overnight at 8 °C with 2% OsO_4 made up with filtered seawater. The supernatant was removed and the cell pellet was allowed to adhere to a coverslip coated with poly-L-lysine (molecular weight 70,000–150,000). Subsequently, cells were washed in Milli-Q water for 10 min and dehydrated through a graded ethanol series (10, 30, 50, 70, 90 and 3× in 100%) for 10 min at each step. The samples were then critical point dried in a K850 Critical Point Dryer (Quorum/Emitech, West Sussex, UK), sputter-coated with gold, and examined with a Zeiss Sigma FE (Carl Zeiss Inc., Oberkochen, Germany) or a Zeiss Ultra 55 FE (Zeiss, Jena, Germany) scanning electron microscope. Images were presented on a black background using Adobe Photoshop CC2014. The standard terminology proposed by Hoppenrath et al. [33] was applied for the description of morphological features, cell orientation and number of platelets.

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