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Molecular phylogeny and toxicity of harmful benthic dinoflagellates *Coolia* (Ostreopsidaceae, Dinophyceae) in a sub-tropical marine ecosystem: The first record from Hong Kong

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ABSTRACT

Coolia are marine benthic dinoflagellates which are globally distributed and potentially toxic. This study provides the first investigation of species diversity and toxicity assessment of *Coolia* in Hong Kong waters. Fifty-one strains of four *Coolia* species, including *C. malayensis*, *C. canariensis*, *C. tropicalis*, and *C. palmyrensis*, were isolated from twelve sub-tidal habitats, and identified phylogenetically using 28S rDNA sequences. Exposure experiments (48-hour) demonstrated that the algal lysates extracted from the four *Coolia* species exhibited different toxic effects on the lethality and abnormality of two invertebrate larvae, i.e., brine shrimp *Artemia franciscana* and sea urchin *Heliocidaris crassispina*. *Heliocidaris crassispina* was more sensitive to the toxic effects of *Coolia* species than *A. franciscana*. Toxicity tests from both larvae revealed that *C. malayensis* was generally more toxic, and caused higher mortality rates when compared with the other three species. The emerging threat of harmful benthic dinoflagellates to marine environments and sensitive biota is discussed.

1. Introduction

Benthic dinoflagellates are a group of marine microalgae distributed worldwide. They are epiphytes that survive on substrate surfaces such as macroalgae, algal turf, seagrasses, coral rubble, rocks, and sediments of shallow tropical and subtropical marine environments (Parsons et al., 2012; Cohu et al., 2013). The geographic ranges and frequency of bloom events for benthic dinoflagellates may further increase due to the warming of seawater under global climate changes (Granéli et al., 2002). Benthic dinoflagellates have attracted considerable attention because of their ability to produce biotoxins that cause food-borne illnesses through the consumption of contaminated seafood, and also lead to other diseases such as respiratory syndromes in humans (Lehane and Lewis, 2000; Gallitelli et al., 2005). More recently, their ecological impact on marine ecosystems, including their association with decreased population fitness and large-scale death of marine organisms, are especially problematic (Shears and Ross, 2009, 2010; Migliaccio et al., 2016).

Benthic dinoflagellate species of the genus *Coolia* are distributed worldwide, and commonly co-exist with other toxic benthic dinoflagellate species from the genera *Ostreopsis*, *Prorocentrum*, *Amphidinium*, and *Gambierdiscus*. These algal assemblages are likely associated with harmful benthic algal blooms and the onset of ciguatera disease (Fukuyo, 1981; Leaw et al., 2016). The following seven *Coolia* species have been identified: *C. monotis* Meunier (Meunier, 1919), *C. tropicalis* Faust (Faust, 1995), *C. areolata* Ten-Hage, Turquet, Quod & Couté (Ten-Hage et al., 2000), *C. canariensis* Fraga (Fraga et al., 2008), *C. malayensis* Leaw, Lim & Usup (Leaw et al., 2010), *C. palmyrensis* Karafas, Tomas & York and *C. santacroce* Karafas, Tomas & York (Karafas et al., 2015). Similar to other benthic dinoflagellates, distinguishing among *Coolia* species based on morphology is difficult because of the minor differences in their external characteristics and the possible occurrence of cryptic species (Momigliano et al., 2013; Leaw et al., 2016). Recent studies have confirmed that the molecular characterization of dinoflagellates using DNA fragments encoding ribosomal RNA genes is particularly useful for taxonomic assessments of *Coolia* species. The most commonly employed regions have been the D1/D2 or D1/D3 regions (Fraga et al., 2008; Mohammad-Noor et al., 2013; Momigliano et al., 2013; Rhodes

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et al., 2014; Karafas and Tomas, 2015; Karafas et al., 2015; Wakeman et al., 2015; Leaw et al., 2016). The *Coolia* species have been genetically well characterized, except for *C. areolata*, with the sequences of multiple strains available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) (reviewed in Leaw et al., 2016).

The toxicity of *Coolia* species is thought to be species specific (Karafas et al., 2015). Strains of *C. malayensis*, *C. palmyrensis*, *C. santacroce*, and *C. tropicalis* were confirmed to be toxic through cytotoxicity tests on a human cell line and bioassays involving intraperitoneal injections of mice and/or exposure to *Artemia franciscana* nauplii (marine invertebrate; Rhodes and Thomas, 1997; Karafas et al., 2015). Toxicity of *C. monotis* has been reported by Rhodes and Thomas (1997) on brine shrimp (*A. salina*) and abalone larvae (*Haliotis virginea*). Toxicity levels differ among *Coolia* species (Laza-Martinez et al., 2011; Rhodes et al., 2014; Karafas et al., 2015), and the biotoxins and bioactive compounds produced by *Coolia* species have not been well characterized. Cooliatoxin is a yessotoxin analog, and was the first identified *Coolia* biotoxin (i.e., in *C. tropicalis* from Australia; Holmes et al., 1995). Another five yessotoxin analogs were recently identified in *C. malayensis* (Wakeman et al., 2015). Other than the limited available information regarding *Coolia* toxic compounds/toxins, little is known about the toxicity of *Coolia* species to various marine organisms.

Hong Kong represents one of the subtropical regions most affected by harmful algal blooms (Smayda, 1990; Lu and Hodgkiss, 2004). Our understanding of local harmful dinoflagellates is primarily limited to the planktonic species, with minimal data available regarding the biodiversity and toxicity of benthic groups. Identifying harmful benthic dinoflagellate species and investigating their toxicity to various marine organisms are important for advancing our understanding of the risks of this group of microalgae to the marine ecosystems in the coastal waters of Hong Kong and the surrounding regions. To the best of our knowledge, this study is the first attempt to characterize the diversity of *Coolia* species from local subtidal habitats using phylogenetic analysis techniques. We aimed to analyze the differential toxicity of various *Coolia* species using invertebrate bioassays. We focused on the larvae of marine organisms as they are particularly susceptible to algal toxic effects. This is because of their increased exposure to toxins through their relatively high metabolic activity and growth rate as well as their lack of an effective enzymatic detoxification system (Vasconcelos et al., 2010). The larvae of a model species commonly used for toxicity studies (i.e., the brine shrimp *A. franciscana*) and a local ecologically important and commercially harvested sea urchin species (i.e., the short-spined sea urchin *Heliocidaris crassispina*; formerly known as *Anthocidaris crassispina*) (Lau et al., 2011) were used in bioassays. Toxicity assessments were conducted using crude algal lysates from eight isolates (i.e., two strains from each of the four *Coolia* species) and the corresponding growth medium solutions (devoid of cells). We assessed the toxicity differences between *Coolia* species and between intra-specific strains. The corresponding responses and sensitivity of the test organisms are discussed.

2. Materials and methods

2.1. Coolia species cultures: collection, isolation, and maintenance

Dinoflagellates from the surface of rocks and dead corals in shallow water (approximately 2–5 m depth below Chart Datum) were collected using a modified bilge pumping method (Parsons et al., 2010), at the east coast of Hong Kong from 2014 to 2016 (Fig. 1 and Supplementary Table S1). Aliquot of lugol (5%) solution fixed samples was used to determine cell density using a Sedgewick-Rafter (Wildlife Supply Company, USA) under an inverted light microscope (Leica DMIRB, Singapore). Single cells of *Coolia* species were isolated from aliquot of live samples using a micropipette under a stereomicroscope (Leica S8APO, Singapore) or an inverted light microscope, and placed in 24-well tissue culture plates (Corning, Kennebunk, USA) with L1 culture medium without silicate (Guillard and Hargraves, 1993). Strains that grew successfully were transferred to sterilized polystyrene culture flasks (Nunc EasYFlasks, Thermo Fisher Scientific, China) containing 25 ml of L1 culture medium for maintenance. Cultures were maintained at 19 °C (±0.5 °C) or 24 °C (±0.5 °C), salinity of 31–32 ppt and irradiance of 47–50 μmol m^{-2} s^{-1} with a 12-h light: 12-h dark cycle inside environmental chambers at the State Key Laboratory in Marine Pollution (SKLMP) of the City University of Hong Kong. Unlike the tropical waters, the local seawater temperature varies with season, the strains collected in winter were therefore maintained at a lower temperature (i.e. 19 °C) while the strains from summer were maintained at a higher temperature (i.e. 24 °C). A total of 51 *Coolia* strains were subjected to phylogenetic analysis in this study.

2.2. Species validation by molecular analysis

2.2.1. DNA extraction and sequencing

Algal cell cultures in the stationary phase were harvested and centrifuged at 3000 rpm for 7 min. The DNA was extracted using the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific, Vilnius, Lithuania). A polymerase chain reaction (PCR) was conducted in a 25-μl reaction containing 20 ng DNA template, 1 unit Ex Taq DNA polymerase (Takara), 0.5 μM reverse and forward primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl). The D1/D3 region encoding the 28S ribosomal RNA gene (28S D1/D3) was amplified using a universal primer pair (D1R and D3Ca) as previously described (Scholin et al., 1993), and the PCR was conducted using a C1000 Thermo Cycler (Bio-Rad, Singapore). PCR was performed as follows: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 1 min; 72 °C for 2 min. Amplicon size and quality were analyzed by 1.5% agarose gel electrophoresis. Amplicons of the expected size were purified and sequenced by Tech Dragon Ltd. (Hong Kong) with the same primer pairs used for the initial PCR.

All sequences of the SKLMP *Coolia* strains were deposited in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov). Accession numbers are listed in Supplementary Table S1.

2.2.2. Alignment and phylogenetic analysis

The DNA sequences of the 28S D1/D3 region were manually edited prior to assembly using Geneious software (version 9.0.2) (Biomatters Ltd.). The alignment of 132 sequences was completed with the MAFFT (version 7.222) plugins of the Geneious program using the default settings of the E-insi algorithm. The aligned dataset included 51 *Coolia* sequences obtained in this study and 81 nucleotide entries for six *Coolia* species and *Gambierdiscus pacificus* (i.e., outgroup) from the NCBI database (Supplementary Table S2). Ambiguously aligned positions were manually excluded, and both ends of aligned sequences were trimmed to produce a uniform fragment length for subsequent phylogenetic analyses. An evolutionary model was constructed for the aligned D1/D3 sequences based on the Akaike Information Criterion using ModelTest (Posada and Crandall, 1998) from the MEGA 6 software (Tamura et al., 2013). The best model was determined as GTR + G + I, and was selected for the subsequent maximum likelihood and Bayesian inference phylogenetic analyses. The maximum likelihood tree was prepared using MEGA 6 with 1000 bootstrap pseudo-replications. The Bayesian inference analysis was conducted using Mr. Bayes 3.2.6 (Huelsenbeck and Ronquist, 2001) in the Geneious program. The posterior probability distribution was estimated using the Markov chain Monte Carlo process with the following settings: random starting tree; two million generations; four heated chains with the temperature set at 0.2; sub-sampling frequency set at 100.

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