Toxicon 56 (2010) 751–758

Contents lists available at ScienceDirect

Toxicon

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

Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 28 April 2009
Accepted 20 May 2009
Available online 28 May 2009

Keywords:
Gambierdiscus
Amphidinium
Coolia
Ostreopsis
Rarotonga
Cook Islands

ABSTRACT

Dinoflagellate species isolated from the green calcareous seaweed, *Halimeda* sp. J.V. Lamouroux, growing in Rarotongan lagoons, included *Gambierdiscus australes* Faust & Chinain, *Coolia monotis* Meunier, *Amphidinium carterae* Hulburth, *Prorocentrum lima* (Ehrenberg) Dodge, *P.* cf. *maculosum* Faust and species in the genus *Ostreopsis* Schmidt. Isolates were identified to species level by scanning electron microscopy and/or DNA sequence analysis. Culture extracts of *G. australes* isolate CAWD149 gave a response of 0.04 pg P-CTX-1 equiv. per cell by an N2A cytotoxicity assay (equivalent to ca 0.4 pg CTX-3C $cell^{-1}$). However, ciguatoxins were not detected by LC-MS/MS. Partitioned fractions of the cell extracts potentially containing maitotoxin were found to be very toxic to mice after intraperitoneal (i.p.) injection. *A. carterae* was also of interest as extracts of mass cultures caused respiratory paralysis in mice at high doses, both by i.p. injection and by oral administration. The Rarotongan isolate fell into a different clade to New Zealand *A. carterae* isolates, based on DNA sequence analysis, and also had a different toxin profile. As *A. carterae* co-occurred with *G. australes*, it may contribute to human poisonings attributed to CTX and warrants further investigation. A crude extract of *C. monotis* was of low toxicity to mice by i.p. injection, and an extract of *Ostreopsis* sp. was negative in the palytoxin haemolysis neutralisation assay.

1. Introduction

Ciguatera has been described since the 1500s, and today is a significant public health problem in many tropical countries world-wide (Dickey, 2008), particularly where coral is found (Laurent et al., 2005). It is widespread in the tropical Pacific and the ecobiology is complex (Mackenzie, 2008). Ciguatera is a shared concern for the Cook Islands and New Zealand, with illnesses attributed to ciguatoxin (CTX) in reef fish occurring in both Cook Island inhabitants (who also have New Zealand citizenship) and New Zealand tourists. Information on the Cook Islands Biodiversity Database (http://cookislands.bishopmuseum.org) states that the first major recorded occurrence of *Gambierdiscus* Adachi & Fukuyo in the region was from the island of Aitu in 1985, and that *Gambierdiscus* is rare in the Northern Group but widespread in the Southern Group.

Gambierdiscus has previously been reported in Northland, New Zealand (Chang, 1996), however with climate change there is the risk of a southward expansion of sub-tropical species into temperate fish aquaculture areas of New Zealand. There is also the potential for the arrival and survival of tropical species into New Zealand coastal waters via ballast water.

This study is our first step into research on biotoxins and their dinoflagellate producers in the Cook Islands, and includes the toxic genera *Gambierdiscus*, and the co-occurring genera, *Amphidinium* Claparède and Lachmann, *Ostreopsis* and *Coolia* Meunier.

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doi:10.1016/j.toxicon.2009.05.017

2. Methods

2.1. Isolation and growth of cultures

Dinoflagellate cells were isolated from the green calcareous seaweed, *Halimeda* sp. (class Chlorophyceae, order Bryopsidales, family Udoteaceae), which was growing on disturbed coral in lagoon sites at lat. 35°: long. 173° (Fig. 1) in June 2007. The micro-algae were collected by shaking the seaweed into contained seawater, then transferring the seawater to 50 ml polypropylene tubes (Falcon™, Becton Dickinson, USA). The tubes were transported by air directly to New Zealand. Samples were fractionated through a series of mesh sieves (>80, >50, >30, >16 μm) and individual cells were picked manually (modified Pasteur pipette) for culture establishment in tissue culture plates (Becton Dickinson, USA) containing f/2 medium (Guillard, 1975). Clones used in this study are maintained in the Cawthron Institute Culture Collection of Micro-algae (CICCM; Table 1).

Preliminary optimisation trials for *Gambierdiscus* isolates indicated an optimal initial pH of 7.8 and light intensity of 40–100 μmol m^{-2} s^{-1}. Nitrate was the optimal N source – cells died in an ammonium chloride based medium. All isolates were maintained under standard conditions of 25 °C, f/2 medium, and 80 μmol m^{-2} s^{-1} (14:10 h light:dark).

Batch cultures of isolates were grown to stationary phase (≈5000 cells per ml) in 5 l Erlenmeyer glass flasks or 10 l plastic barrels (standard conditions) with filtered air (0.45 μm) pumped through the medium.

2.2. Identification

2.2.1. Scanning electron microscopy (SEM)

Cells were fixed in a glutaraldehyde (3%), formaldehyde (2%), phosphate buffer (0.1 M) solution, then passed through an ethanol series (75%, 50%, 25%, distilled H_2O; 1 h each dilution). The cells were then sandwiched between two polycarbonate filters, critical point dried and gold coated for SEM (FEI Quanta 200).

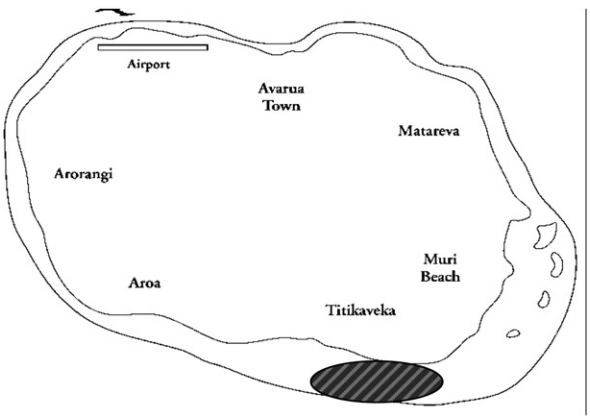

Fig. 1. Map of Rarotonga, Cook Islands, with striped area highlighting the main sampling sites.

Table 1
Dinoflagellate species cited in this study and maintained in the Cawthron Institute Culture Collection of Micro-algae.

Species	Code	Source country
Amphidinium carterae	D152	Cook Islands
Amphidinium carterae	D22,23,57	New Zealand
Coolia monotis	D151	Cook Islands
Coolia monotis	D39,60,77,98	New Zealand
Gambierdiscus australes	D149	Cook Islands
Ostreopsis sp.	D150	Cook Islands
Ostreopsis siamensis	D75,146,97	New Zealand
Prorocentrum lima	D157	New Zealand
P. cf. *maculosum*	D158	New Zealand

2.2.2. DNA extraction, PCR amplification and sequencing

Exponentially growing culture (10 ml) was harvested by centrifugation (542 × *g*) for 15 min at room temperature, and the pellet transferred to a 1.5 ml tube and frozen overnight (−20 °C). Total genomic DNA was extracted using the DNeasy mini plant kit (Qiagen, California, USA). The D1–D3 fragment of the large subunit ribosomal RNA (LSU rRNA) gene was amplified using the primers D1R-F (Scholin et al., 1994) and D3B-R (Nunn et al., 1996). All PCR reactions were performed using an iCycler (BioRad, California, USA): 50 μl reaction mixes contained 45 μl of PCR supermix (Invitrogen, California, USA), 2 μl of each primer (10 μM), and 1 μl of DNA (conc. 20–100 ng per reaction). PCR conditions were 95 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 2 min, and a final extension of 72 °C for 6 min. PCR products were checked by gel electrophoresis through 1.5% (w/v) agarose gels. Positive PCR products were purified with QIAquick PCR purification kit (Qiagen, California, USA). Purified amplicons were sequenced at the Waikato DNA Sequencing Facility, Hamilton, New Zealand, in both directions.

2.2.3. Phylogenetic analyses

Sequence chromatograms were examined visually and base-calling errors corrected manually. Both forward and reverse sequences were aligned and conflicts resolved by manual inspection. Corrected consensus sequences were aligned with sequences from GenBank using the ClustalW algorithm in BioEdit sequence alignment editor (Hall, 1999). Bayesian analyses were carried out using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), using the generalised time reversible model with a proportion of invariable sites and a gamma shaped distribution of rates across sites. A uniform clock model was enforced. The Bayesian analyses were carried out in two simultaneous runs for 1.5×10^5 generations, with four chains each. The trees were sampled every 100 generations. Of the 1500 trees sampled the latter 1000 were used to construct a 50% majority-rule consensus tree.

2.3. Extraction of Gambierdiscus

Gambierdiscus australes cells, isolate CAWD149, in stationary phase (1 l culture, 7.0×10^5 cells; 15 l culture, 1.2×10^7 cells) were separated into cell and supernatant by settling and centrifugation (3000 × *g*, 4 min, room temperature; 50 ml Falcon tubes). Cell pellets were