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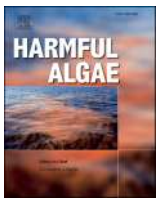

Original Article

Diversity and toxicity of Pacific strains of the benthic dinoflagellate *Coolia* (Dinophyceae), with a look at the *Coolia canariensis* species complex

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ABSTRACT

Coolia Meunier 1919 from benthic assemblages of Hawai'i and Guam were isolated and clonal cultures were established from single cells. Cultures were identified to species-level based on 28S rRNA and ITS-5.8S rRNA genes and tested for toxicity. In Hawai'i, two strains of *C. malayensis* were isolated. In Guam, a high biodiversity was identified: four strains of *C. malayensis*, one strain of *C. palmyrensis*, one strain of *C. tropicalis*, one strain of *C. canariensis* phylogroup III, and two strains forming a new phylogroup (phylogroup IV) of nontoxic *C. canariensis*. Morphology of the new *C. canariensis* phylogroup was described using light microscopy and scanning electron microscopy. Mass cultures and methanol extracts of representative cultures (*C. malayensis, C. palmyrensis, C. canariensis, C. tropicalis*) from Guam were prepared for liquid chromatography-mass spectrometry analysis. Chemical analyses revealed yessotoxin analogue $C_{56}H_{78}O_{18}S_2$ is produced by *C. malayensis, C. canariensis phylogroup IV* and *C. palmyrensis*, but other analogues, $C_{57}H_{80}O_{18}S_2$ and $C_{58}H_{86}O_{18}S_2$, were only found in *C. malayensis* (Okinawa) and *C. canariensis* phylogroup IV. Individual toxin profiles were also different over time for an Okinawa strain of *C. malayensis* (NIES-3637), highlighting intra and inter-species variation in Yessotoxin-analogue expression. Biological activity was tested using *Artemia* bioassay and toxicity was observed in Guam and Okinawa strains of *C. malayensis*. Strong support of four distinct clades within the *C. canariensis* species complex was recovered in phylogenetic analyses, despite morphological similarities.

1. Introduction

Marine benthic dinoflagellates produce natural products that may be toxic and can have negative implications for marine organisms and public health (Wang, 2008; Moestrup et al., 2014; Longo et al., 2020; Mudadu et al., 2021). One such genus, *Coolia* Meunier 1919, has a broad distribution, ranging from tropical to temperate waters (Gómez et al., 2016; Leaw et al., 2016), and some strains are known to produce toxic compounds (e.g., cooliatoxin) (Holmes et al., 1995; Leaw et al., 2016). Currently, eight species of *Coolia* are recognized (David et al., 2020); four of these lineages, *C. tropicalis, C. malayensis, C. palmyrensis*, and *C. santacroce* are known to be toxic (Holmes et al., 1995; Rhodes and Thomas, 1997; Laza-Martinez et al., 2011; Karafas et al., 2015). Still, our contemporary understanding of morphology within the genus, and the use of molecular phylogenetics (mainly based on 28S and ITS-5.8S regions of the ribosomal RNA operon), have resulted in the identification of species complexes within *Coolia* (Nascimento et al., 2019; David et al., 2020). It is likely that within this genus there is an unexplored diversity that has yet to be teased apart, and to this end, *Coolia* is an intriguing model to study the biodiversity and toxin production of marine benthic dinoflagellates.

Coolia monotis Meunier 1919 is the type species for this genus. Other species within this genus have been differentiated by variation in the thecal plate pattern such as the shape of the 1′ and 3′ plates in *C. tropicalis, C. malayensis* and *C. canariensis* (Meunier, 1919; Faust, 1995; Fraga et al., 2008; Leaw et al., 2010), areolation in *C. areolata* (Ten-Hage et al., 2001), or pore size in *C. guanchica* and *C. palmyrensis* (Karafas et al., 2015; David et al., 2020). The more contemporary use of DNA sequencing, has facilitated the identification of more species that appear morphologically stagnant, or have phenotypic plasticity (Laza-Martinez et al., 2011; Leaw et al., 2016).

Molecular phylogeny of the 28S and ITS-5.8S rRNA gene from

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C. canariensis recovered three separate phylogroups with strong nodal support (David et al., 2014; Nascimento et al., 2019). Phylogroup I has only been found in the Canary Islands (Fraga et al., 2008); phylogroup II only in Trinidad Island, Brazil (Nascimento et al., 2019); phylogroup III has been found in multiple locations including Hong Kong (Leung et al., 2017), South Korea (Jeong et al., 2012), Spain (Fraga et al., 2008; Laza-Martinez et al., 2011; David et al., 2014) and Australia (Momigliano et al., 2013). However, these geographic boundaries may be the result of limited sampling effort. The ITS sequence for phylogroup I is not available, however, pairwise comparison (*p*-distance) between phylogroup II and III is $\geq$ 0.04, which is greater than the genetic differences between other recognized *Coolia* species (i.e., *C. monotis, C. malayensis* and *C. tropicalis*) (Litaker et al., 2007; Nascimento et al., 2019). Despite this genetic variation, there is no consistent and reliable morphological character unique to each *C. canariensis* phylogroup.

Certain strains of *C. malayensis, C. palmyrensis, C. santacroce* and *C. tropicalis* produce natural products that are toxic such as cooliatoxin, a polyether toxin analogous to yessotoxin (Holmes et al., 1995), and gambierone (Tibiriçá et al., 2020), which can be toxic to mice, *in vitro* human cell lines and invertebrates (Rhodes and Thomas, 1997; Rhodes et al., 2010; Karafas et al., 2015; Leung et al., 2017). One strain of *C. malayensis* produces several analogues of yessotoxin (Wakeman et al., 2015). While *C. malayensis, C. palmyrensis, C. santacroce* and *C. tropicalis* are generally known to produce toxins, some intraspecies variation exists. For example some strains of *C. malayensis*, and *C. tropicalis* are non-toxic (Rhodes et al., 2014; Leaw et al., 2016).

The aim of this study was to understand the diversity and toxicity of *Coolia* from Guam and Hawai'i; two under-sampled regions in the Pacific Ocean. *Coolia* was isolated into clonal cultures from shallow reefs from both locations and identified to species level based on 28S and ITS-5.8S rRNA genes. Their chemical profiles and toxicity were also evaluated using HPLC and *Artemia* bioassays, respectively. An existing culture of *C. malayensis* (NIES-3637) was also examined, in order to gain a perspective into the intra-species/intra-strain variation of the chemical profiles.

2. Materials and methods

2.1. Sample collection and treatment

Seawater samples were collected from macroalgae and turf algae on coral reefs in Pago Bay, Guam (13°25′39.6" N 144°47′54.1" E) and Waimanalo, Hawai'i (21°19′52.29'' N 157° 41′12.40'' W). Samples were bottled and brought back to the laboratory then separated into plastic containers with half-concentration DAIGO IMK, and incubated for two weeks at 25 °C. Single cells of *Coolia* were isolated and grown in 48-well plates in half-concentration DAIGO IMK growth medium. After the cultures became established (and cell density was visible by eye), they were transferred to a Petri dish containing DAIGO IMK.

Coolia malayensis from Okinawa is the strain referenced in Wakeman et al. (2015), acquired from NIES microbial culture collection (NIES-3637).

2.2. Morphological analysis

Cultured cells were fixed with 2.5% glutaraldehyde then washed with seawater. The cells were then permeabilized at 20 °C for 30 min with an enzyme mix of 1% hemicellulose (Sigma-Aldrich, USA), 1% cellulase (MP Biomedicals, California, USA) and 0.4 M mannitol (Wako Pure Chemical Industries, Osaka, Japan), adjusted to pH 5.8 and stained with 0.1 μg/mL DAPI (Roche Diagnostics GmbH, Germany) then imaged with Nikon eclipse Ti2 (Nikon, Tokyo, Japan). For scanning electron microscopy (SEM), 1 ml of cell culture was fixed with 6% glutaraldehyde overnight then filtered through a 10 μm MF-Millipore™ (Merck, USA) membrane filter. Cells were then washed twice with distilled water and dehydrated through five steps with increasing ethanol concentration (60%, 70%, 80%, 90%, and three times 100%) for 5 min at each step and left to air-dry overnight. They were then mounted on stubs and sputter-coated for 4 min with 5 μm gold and viewed using Hitachi N-3000 scanning electron microscope (Hitachi, Tokyo, Japan).

2.3. DNA extraction, PCR amplification and sequencing

Extraction of genomic DNA was performed on 5 cells in PCR tubes containing 10 μl QuickExtract™ FFPE DNA Extraction Kit (Lucigen corp., USA) and incubated at 56 °C for 1 h then 98 °C for 2 min. To identify cultures at the species level, 28S rRNA (28S) and ITS1-5.8S-ITS2 (ITS) genes were amplified and sequenced. Initial amplification of 18S, ITS, and 28S rRNA genes was done using primers PF1 ~ 28-1483R (Daugbjerg et al., 2000; Leander et al., 2003) with MyFi™ Mix and the following program on a thermocycler: initial denaturation 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 52 °C for 15 s, 72 °C for 3 min 30 s; final extension 72 °C for 7 min. Then 1:100 dilution of the initial PCR reaction was used as template for a nested reaction using primer set D1RF1 ~ 25R1 (D1 region) and 305-27F ~ 852R -70 (D2 region) (Yamada et al., 2013) using EconoTaq 2 × Master Mix (Lucigen corp., USA), with the following program on a thermocycler: initial denaturation 94 °C for 2 min; 25 cycles of 94 °C for 15 s, 52 °C for 15 s, 72 °C for 1 min 40 s; final extension 72 °C for 7 min. To amplify the ITS region, initial reaction using primer set SR4 ~ 25R1 (Takano and Horiguchi, 2006; Yamaguchi et al., 2006) with MyFi™ Mix and the following program on a thermocycler: initial denaturation 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 52 °C for 15 s, 72 °C for 3 min; final extension 72 °C for 7 min. Then 1:100 dilution of that reaction was used as template for a nested reaction using primer set Lp1F1 ~ 25F1R (Howard et al., 2009; Takano and Horiguchi, 2006) using EconoTaq 2 × Master Mix (Lucigen corp., USA), with the following program on a thermocycler: initial denaturation 94 °C for 2 min; 25 cycles of 94 °C for 15 s, 52 °C for 15 s, 72 °C for 1 min 40 s; final extension 72 °C for 7 min. Supplementary table 1 lists the primer sequences.

Size of PCR products was checked on 1% agarose gel, purified with Polyethylene Glycol (PEG), dye-labeled with BrilliantDye™ Terminator v1.1 (Nimagen, Netherlands) according to manufacturer's protocol, and sequenced on a 3130 genetic analyzer (Applied Biosystems, Massachusetts, USA). The sequences were checked using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST). Sequences generated in this study were deposited in the NCBI under the following accession numbers: MZ801746-MZ801769.

2.4. Phylogeny

The 28S and ITS rDNA sequences obtained in this study were aligned using the MAFFT E-INS-I algorithm online (Katoh et al., 2002) along with *Coolia* sequences retrieved from GenBank and the alignments were manually checked in Mesquite (Maddison and Maddison, 2019). Alignments were trimmed using Gblocks, Version 0.91b (Castresana, 2000; Talavera and Castresana, 2007) with less stringent selection options; allow smaller final blocks, allow gap positions within the final blocks, allow less strict flanking positions. Each locus was aligned separately and *Ostreopsis ovata* was used as an outgroup. The final 28S alignment contained 100 sequences with 741 aligned nucleotides, and the ITS rRNA alignment contained 72 sequences with 311 aligned nucleotides (Supplementary file 1 and 2).

Iqtree 1.6.12 software was used to choose the best substitution model according to Akaike Information Criterion with correction (Nguyen et al., 2014). Substitution model TIM+F+G4 and TN+F+G4 were used for 28S and ITS alignments, respectively. Maximum likelihood (ML) tree inference was run in Iqtree with 1000 bootstrap pseudoreplicates.

Bayesian analysis was performed with MrBayes v3.2.7 (Ronquist and Huelsenbeck, 2003). The General Time Reverable model with a gamma distribution of site-rate heterogeneity was specified. Four Monte-Carlo-Markov chains were run for 1,000,000 generations and

sampled every 100th generation. Tracer 1.7 (Rambaut et al., 2018) was used to check convergence and posterior probability values were obtained from the consensus tree, after the first 25% was discarded as burn-in.

MEGA-X (Kumar et al., 2018) was used to calculate the pairwise distances between the phylogroups for each the 28S and ITS genes. The model selected was p-distance with transition and transversions; evolutionary rates among sites were set as gamma-distributed. Other parameters were left at default settings.

2.5. Methanol extraction and desalting of crude extracts

Representative strains (NIES-3637, G10, F6-G23, G6, G11, G8) were grown in mass cultures for toxin analysis. Cells were filtered through Whatman® (Merck, USA) filter paper grade 1 and stored in 50 ml falcon tubes in -20 °C until ample material had been collected. Frozen tubes were thawed for 30 min at room temperature then 25 ml methanol was added to each falcon tube, vortexed and sonicated for 5 min. After which the samples were left at room temperature for 30 min with occasional shaking then transferred into another flacon tube. Extraction was repeated another two more times, the last extraction was left overnight at room temperature. The methanol extractions were centrifuged, decanted and concentrated using SP Genevac EZ-2 Plus (SP Scientific, UK) until each sample contained about 5 ml solution. The extract was defatted by adding 5 ml ethyl acetate and vortexed for 10 min before separating water-soluble and ethyl-acetate soluble parts. Defatting by addition of ethyl acetate and separation water-soluble and ethyl-acetate soluble parts was repeated two more times. A portion of water-soluble part was desalted using ZipTip NIES-36378/P10 (Millipore) and the remaining dried using the evaporator and stored at -20 °C and later desalted using Supel™-Select HLB 1ml tubes (Merck, USA).

Zip tips were activated using aqueous-methanol-formic acid (1:9:0.05) solution. Concentration of methanol was decreased with each step (90%, 50%, 10%), waiting 2 min between each step. Sample was passed through the tip 10-15 times then washed with aqueous-methanol-formic acid (9:1:0.05). The retained sample was eluted with 10 µl aqueous-methanol-formic acid (1:9:0.05) into HPLC sample tubes and diluted by adding 20 µl aqueous-methanol-formic acid (9:1:0.05).

Solid phase extraction tubes, 1 ml HLB tubes (30 mg/ml) were activated using 1 ml MeOH then 1 ml aqueous-methanol-formic acid (9:1:0.05). Dried water-soluble part was dissolved using aqueous-methanol-formic acid (9:1:0.05) then passed over the HLB column and washed with 1 ml aqueous-methanol-formic acid (9:1:0.05) two times. Finally, the retained sample was eluted with 1 ml MeOH (30 mg/ml), dried in the evaporator and stored in -20 °C.

2.6. Nanoliquid chromatography mass spectrometry

Water-soluble part was dissolved in 200 µl MeOH and diluted 1:10 in aqueous-methanol-formic acid (9:1:0.05) and loaded into a microcolumn-Liquid Chromatograph (Paradigm MS4, Microm Bioresources) connected to a Thermo Scientific Hybride Mass Spectrometer (LTQ Orbitrap) for mass spectrometry (MS) data collection. The mass spectrometer parameter was set up following Wakeman et al. (2015).

2.7. Artemia lethality bioassay

Artemia eggs were placed in 0.22 µm-filtered seawater for 24 h to hatch. Ten nauplius were transferred into a 24 well plate containing 475 µl filtered seawater and 25 µl *Coolia* culture extract water-soluble fraction. Three wells (n = 30) were made for each *Coolia* extract. Water-soluble part from solid phase extraction HLB tubes was dissolved in 100 µl distilled water (Invitrogen, USA) and 25 µl was added to each well. Negative controls were seawater and 25 µl distilled water. Viability of *Artemia* nauplius was checked at intervals of 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 30 h, and 48 h.

3. Results

3.1. Morphological analyses C. canariensis phylogroup III and IV from Guam

Coolia canariensis phylogroup IV isolated from Guam have an average dorsal-ventral (DV) width of 28.78 ± 3.4 µm (range = 22 – 34 µm, n = 28) and DV depth of 30.8 ± 3.3 µm (range = 23 – 37 µm, n = 21). The nucleus is bean-shaped, central in the cell (Fig. 1). The plate formula is Po, 1′, 7″, ?c, 5″, ?s, 2‴′. The surface of the cell is smooth, compared to the rough, ornamented hypotheca (Fig. 2A). The apical pore is elongated and slightly curved, average length was 6.80 ± 0.83 µm (range = 5.21 – 7.75 µm, n = 9) and width is 1.53 ± 0.44 µm (range = 0.843 – 2.17 µm, n = 8), connected to plates 1′, 2′ and 3′ (Fig. 2B). The 1′ plate is the largest epithecal plate, hexagonal and connected to Po, 2′, 3′, 1″, 2″, 6″ and 7″ plates. Plate 2′ is thin rectangular, touching the Po, 1′, 2″, 3″ and 4″ plates. Plate 3′ is pentagonal, contacting Po, 1′, 2′, 4″, 5″ and 6″ plates (Fig. 2B). Plate 1‴ is trapezoidal, touching 1′, 2″ and 7″ (Fig. 2B). Plate 2″ is pentagonal, elongated at the cingulum and is contacting 1′, 2′, 1″ and 3″ plates (Fig. 2C). Plate 3″ is rectangular, touching 2′, 2″ and 4″ plates (Fig. 2D). Plate 4″ is square, touching 2′, 3′, 3″ and 5″ plates (Fig. 2D). Plate 5″ is trapezoidal, contacting 3′, 4″ and 6″ plates (Fig. 2D, E). Plate 6″ is pentagonal, touching 1′, 3′, 5″ and 7″ plates, the shortest side is contacting 3′ plate (Fig. 2E). Plate 7″ is trapezoidal, contacting 1′, 1″ and 6″ plates; it also extends further than plate 1″ making an uneven cingulum (Fig. 2A, E). Postcingulum plate 1‴ is triangular, contacting plate 2‴ and 1⁗ (Fig. 2A). Plate 2‴ is trapezoidal, touching plates 1‴, 3‴ and 2⁗ (Fig. 2A, B, F). Plates 3‴ and 4‴ are trapezoidal touching plates 2‴, 4‴, 2⁗ and 3‴, 5‴, 2⁗ respectively (Fig. 2F). Plate 5‴ is triangular contacting plate 4‴ (Fig. 2F). Of the post cingulum plates, Plate 3‴ is smoothest, 1‴ and 5‴ have the roughest surface followed by plates 2‴ and 4‴ (Fig. 2F). Plate 1⁗ is protruding over the sulcal plates contacting plate 1‴ (Fig. 2F). Plate 2⁗ is a small square surrounded by plates 1‴, 2‴, 3‴, 4‴ and Sp (Fig. 2F).

Coolia canariensis phylogroup III from Guam and *C. canariensis* phylogroup IV show the same plate morphology (Fig. 3A - F). It has roughly spherical cells with a plate formula Po, 1′,7″, ?c, 5″, ?s, 2⁗, average DV width 28.17 ± 1.72 µm (range = 25 – 38 µm, n = 6) average DV depth = 28.0 ± 2.65 µm (range = 25 – 31 µm, n =7) (Fig. 3A - F). Apical pore is surrounded by plates 2′, 3′, 1′, and is elongated and slightly curved with average length = 7.22 ± 0.91 µm (range = 8.46 – 5.92, n = 5) and average width = 1.31 ± 0.39 µm (range = 0.86 – 1.81, n = 4) (Fig. 3B).

3.2. Phylogenetic analyses

Both analyses of 28S and ITS regions recovered seven distinct clades representing seven species (Fig. 4 and 5). Strains G4, G10, E6-G27, E2G25, NIES-3637, C12, C14 from Guam and Hawai'i grouped with *C. malayensis*. Strains F6-G23 and G8 from Guam grouped with *C. palmyrensis* and *C. tropicalis*, respectively. Based on the 28S

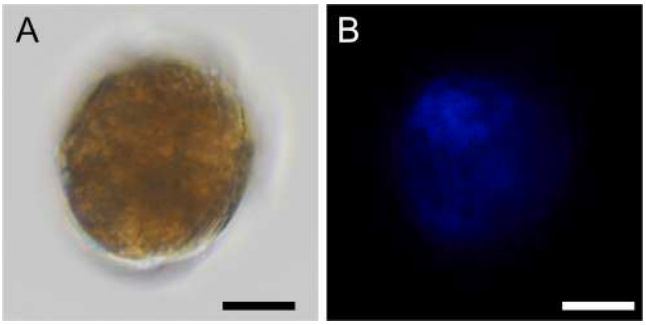

Fig. 1. A) Light microscopy and B) epifluorescence image of *Coolia canariensis* phylogroup IV. Scale bar–10 µm.

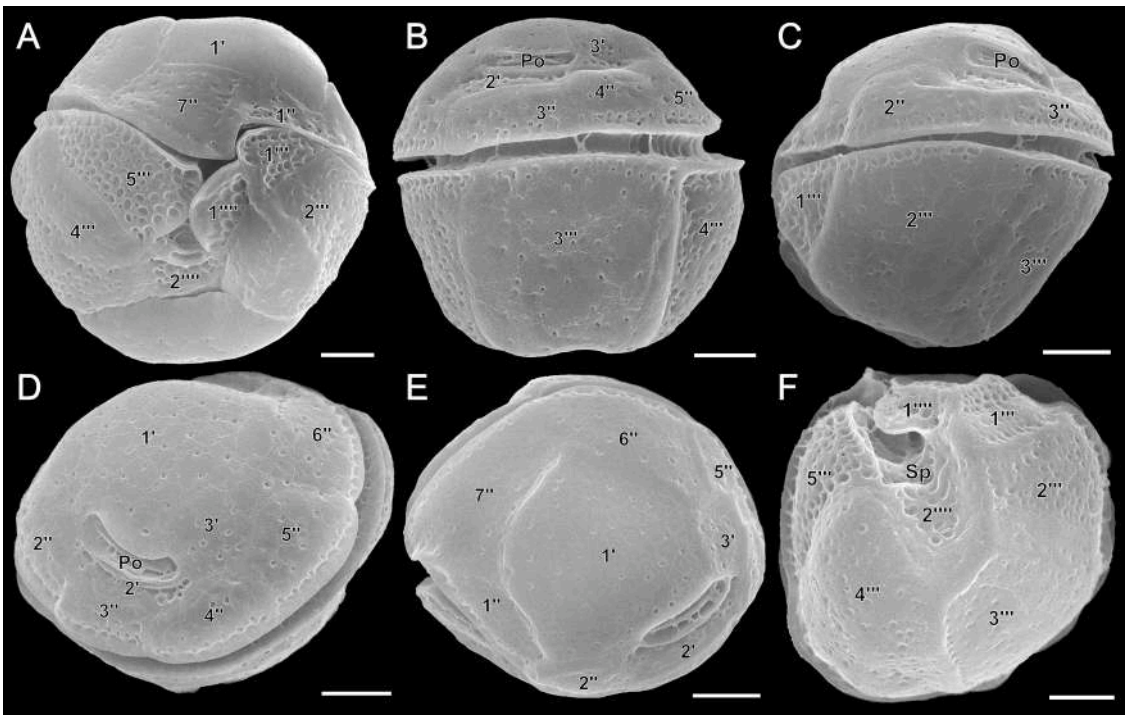

Fig. 2. Scanning electron micrographs of *Coolia canariensis* phylogroup IV. A) Ventral view. B) Dorsal view. C) Antapical view. D) Apical view. E) Apical right-lateral view. F) Right lateral view. Apical pore (Po), Sulcal plate (Sp). Scale bars–5 μm.

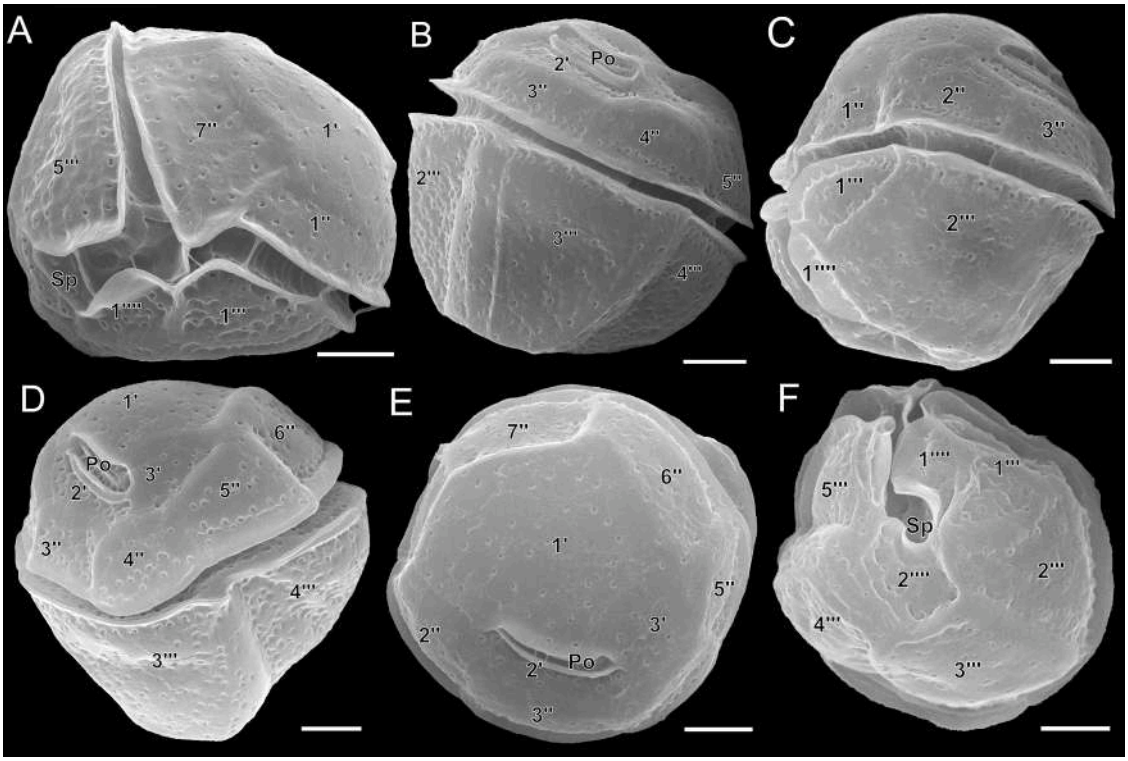

Fig. 3. Scanning electron micrographs of *Coolia canariensis* phylogroup III isolated from Guam. A) Ventral view. B) Dorsal view. C) Antapical view. D) Apical view. E) Left leteral view. F) Right leteral view. Apical pore (Po), Sulcal plate (Sp). Scale bars–5 μm.

phylogeny, the *C. canariensis* clade contains four phylogroups with moderate-to-high bootstrap and Bayesian posterior probability (≥70 / ≥0.95). Phylogroup IV consists of two strains from Guam (G6 and G17), and was sister to phylogroup III (Fig. 4). The ITS phylogeny of *C. canariensis* clade consisted of six sequences and did not include phylogroup I (Fig. 5). Strain G6 and G17 were grouped with high bootstrap values and Bayesian posterior probability (100/1) in phylogroup IV, sister to phylogroup III. Strain G11 from Guam did not show strong support as a member of phylogroup III (Fig. 5). Pairwise comparison of 28S rRNA gene showed the average p-distance within each

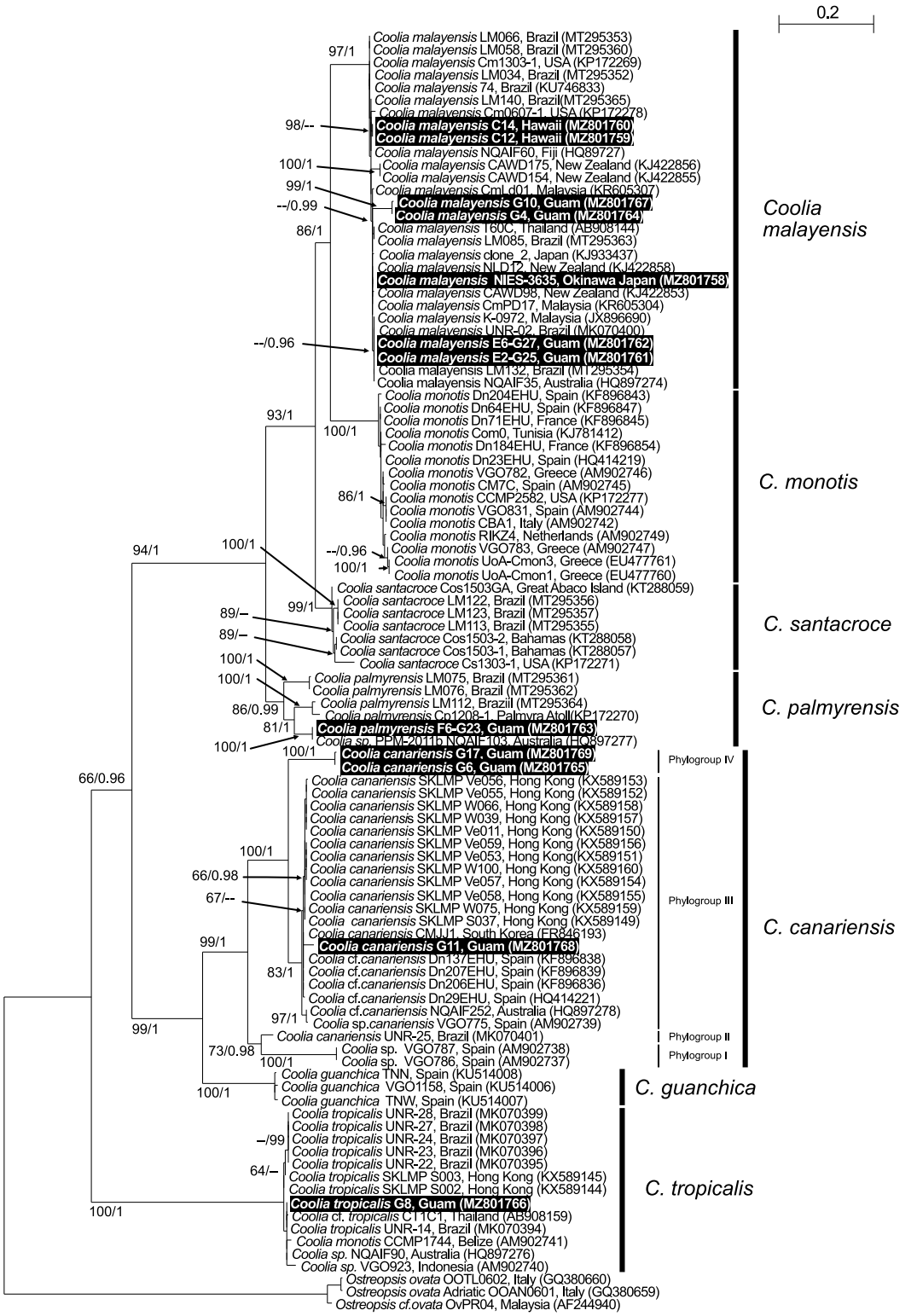

Fig. 4. Maximum likelihood phylogenetic tree inferred from 28S sequences using TIM F G4 substitution model. Bootstrap support lower than 60% and Bayesian posterior values lower than 95% are not displayed. Sequences published from this study are written in white, bold and highlighted.

species was highest in *C. canariensis* (p = 0.079 substitutions per site), followed by *C. palmyrensis* (p = 0.0718); the remaining *Coolia* species ranged between p = 0.0016 - 0.0138 (Supplementary table 2). Mean distance between species was shown to be less between *C. monotis, C. malayensis* and *C. santacroce* (p = 0.106 - 0.148), than phylogroups of *C. canariensis* and strains G6 and G17 (p > 0.15) (Supplementary table 3). This was also matching in p-distance calculated for ITS, where *C. canariensis* strain G6 and G17 was p > 0.3 in relation to strain G11, and previously studied phylogroups II and III (Supplementary table 4).

3.3. Mass profile of Coolia culture extract

High-resolution mass (HRMS) was used to identify cooliatoxin in water-soluble part which were previously identified toxins by Wakeman

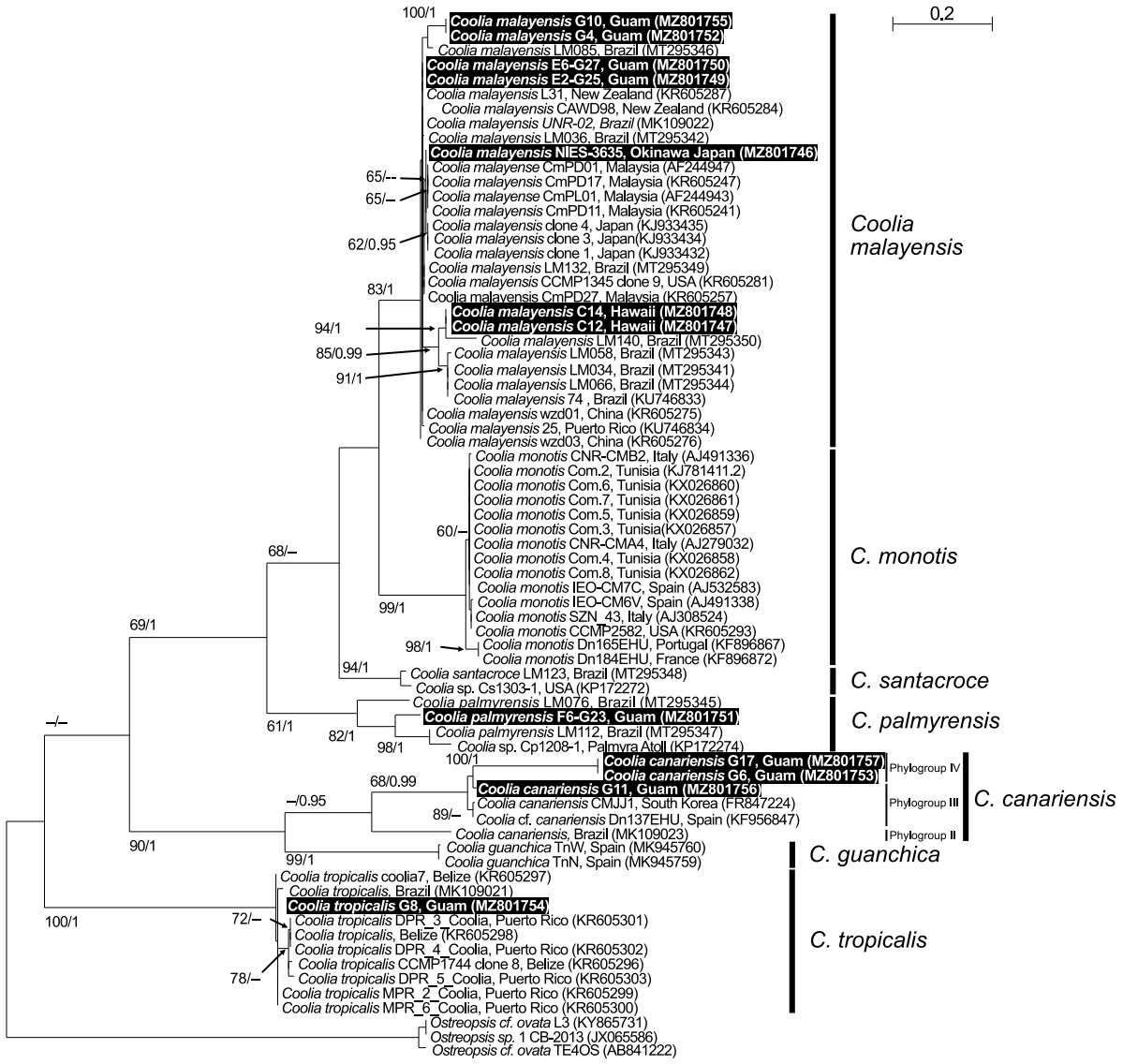

Fig. 5. Maximum likelihood phylogenetic tree inferred from ITS sequences using TN F G4 substitution model. Bootstrap support lower than 60% and Bayesian posterior values lower than 95% are not displayed. Sequences published from this study are written in white, bold and highlighted.

et al. (2015). *Coolia malayensis* strain NIES-3637 used was the same strain as the previous study, cooliatoxin **1** and **2** at *m/z* 1101.46 $C_{56}H_{78}O_{18}S_2$ $[M-H]^-$ Δ 5.14 mmu, and 1115.47 $[M-H]^-$ Δ 2.43 mmu respectively were identified (Supplementary fig. 2A, B). MS^2 fragmentation of toxin compound **1** showed the sulphonate loss $[M-SO_3-H]^-$ at *m/z* 1021.41, MS^3 fragmentation showed water loss $[M-SO_3-H_2O-H]^-$ at *m/z* 1003.50 (Supplementary fig. 2D - G). Due to low signal intensity, the peak for toxic compound **2** at *m/z* 1115.47 was not selected for MS^2 fragmentation (Supplementary fig. 2G).

From strain G10 from Guam, compound **1** at *m/z* 1101.45, $C_{56}H_{78}O_{18}S_2$ $[M-H]^-$ Δ 1.42 mmu and compound **5** *m/z* 1133.5213 $C_{58}H_{86}O_{18}S_2$ $[M-H]^-$ Δ 4.08 mmu were detected (Fig. 6). In *C. palmyrensis* strain F6-G23, a peak at *m/z* 1101.45 $C_{56}H_{78}O_{18}S_2$ $[M-H]^-$ Δ 1.467 mmu (Fig. 7) and *C. canariensis* phylogroup IV strain G6 *m/z* 1101.45, $C_{56}H_{78}O_{18}S_2$ $[M-H]^-$ Δ 2.66 mmu, *m/z* 1133.52 $C_{58}H_{86}O_{18}S_2$ $[M-H]^-$ Δ 9.14 mmu were detected (Fig. 8). In these three strains, MS^2 fragmentation was not observed due to less intense signal. Peaks matching the toxin compounds were not detected in *C. canariensis* phylogroup III (Strain G11) nor *C. tropicalis* (Strain G8).

3.4. Artemia lethality bioassay

There was no visible change for the first 8 h after addition of the water-soluble part. After 12 h, *Artemia* with the addition of *C. malayensis* extracts (NIES-3637, Okinawa and G10, Guam) had impaired mobility. After 24 h, *Artemia* with NIES-3637 extracts were alive however motility severely diminished. *Artemia* nauplius in wells with G10 extracts had 10% mortality and severely diminished mobility (Supplementary video 1). After 30 h, *Artemia* with extracts from NIES-3637 were no longer capable of swimming, and similar to G10, had a 33% mortality rate. After 48 h, mortality was detected for NIES-3637 (avg. death = 36%), majority of *Artemia* with G10 extracts were dead (avg. death = 93%). Toxic effects of the water-soluble fraction on *Artemia* for *C. palmyrensis* (F6-G23) and *C. canariensis* (G6, G11) were not observed.

4. Discussion

4.1. Global distribution of Coolia

In the present study, 11 strains of *Coolia* from Guam and Hawai'i were established. Despite our limited geographical sampling, a larger

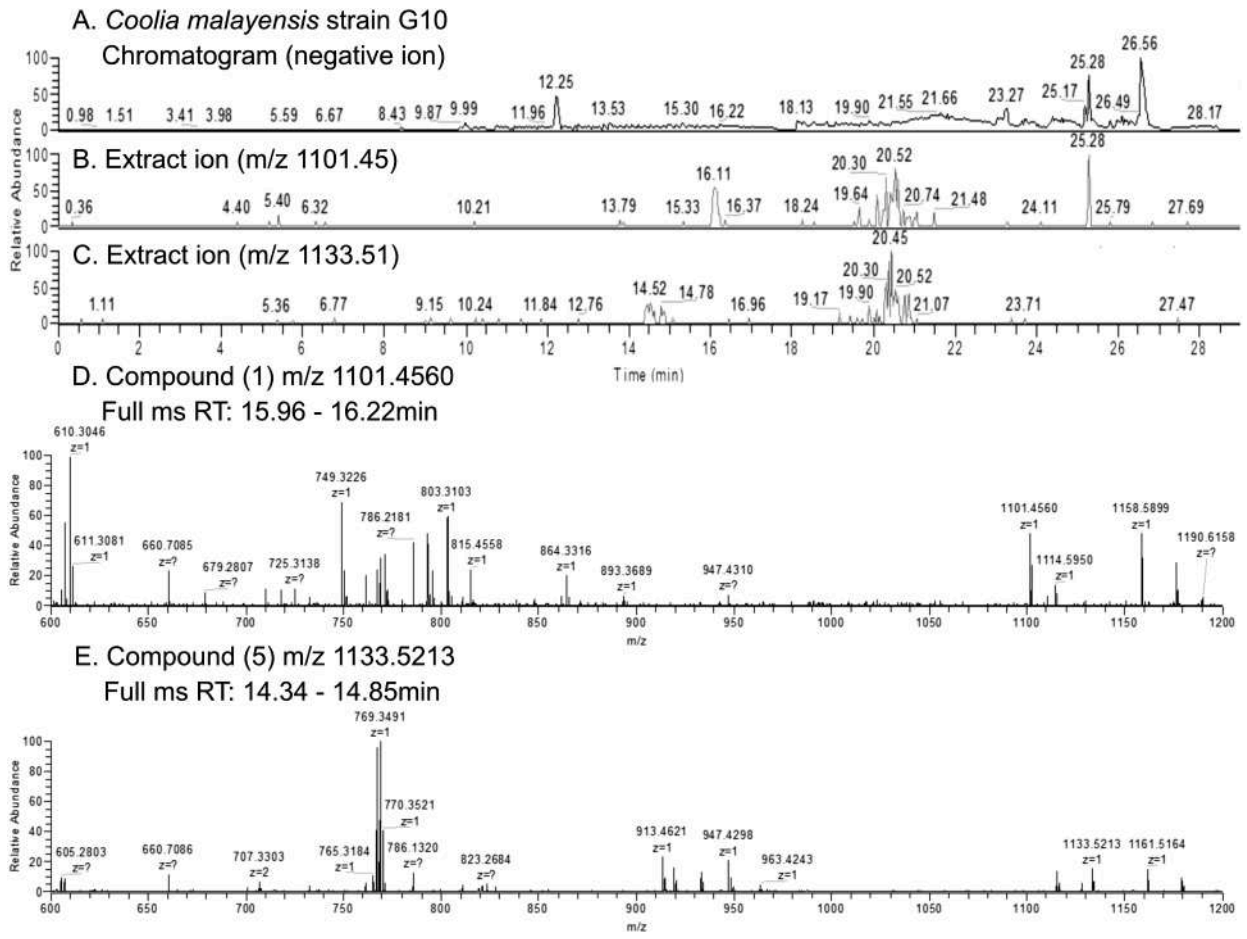

Fig. 6. NanoLCMS (negative ion) profile of water-soluble extract of *Coolia malayensis* strain G10. A) Chromatogram; B) extract ion (*m/z* 1101.45); C) extract ion (*m/z* 1101.45); D) compound 1 (*m/z* 1101.4560, RT–15.96-16.22 min); E) compound 5 (*m/z* 1133.5213, RT–14.34 – 14.85 min).

than expected diversity of *Coolia* was found. Phylogenetic analysis of 28S and ITS rRNA genes of strains from Pago Bay, Guam, grouped into four of the eight known species (*C. malayensis C. palmyrensis, C. tropicalis* and *C. canariensis*). The high diversity of *Coolia* species is similar to previous studies in Hong Kong (Leung et al., 2017), Great Barrier Reef (Momigliano et al., 2013), and Brazil waters (Tibiriçá et al., 2020), indicating a broad distribution of *Coolia*. This supports the idea that many species within the *Coolia* genus have overlapping geographic ranges, and suggests a continuous distribution, and rich diversity of *Coolia*, throughout the Western Pacific. Conversely, the distribution of *C. monotis* has only been isolated from Europe, the Mediterranean Sea and North Atlantic, which is evidence that some species do have restricted distributions, possibly due to habitat restrictions like temperature (Karafas et al., 2015; Lewis et al., 2018).

4.2. Examining the phylogeny and diversity of C. canariensis species complex

This study also found a new non-toxic phylogroup within the *C. canariensis* species complex (phylogroup IV). The morphology of strains G6 and G17, which formed phylogroup IV, was similar to strain G11 (phylogroup II) and other *C. canariensis* phylogroups. Generally speaking, *Coolia* have been morphologically characterized by their thecal plate patterns and other ornamentations of the thecal plates. *Coolia monotis*, for example, has a 6″ plate which is the largest epitheca plate, shifting the 1′ plate to the left of center. This is distinct from *C. tropicalis, C. areolata, C. guanchica* and *C. canariensis* where the 1′ plate is located centrally on the epicone. These morphological characteristics have been supported by molecular data (Faust, 1995; Fraga et al., 2008; Leaw et al., 2010; Mohammad-Noor et al., 2013; Karafas et al., 2015). *Coolia tropicalis* has a smooth thecal surface, differentiating it from *C. areolata* which is heavily areolated (Ten-Hage et al., 2001). The epitheca of *C. canariensis* is smooth but the hypotheca is ornamented prominently in postcingular plates 1‴, 2‴, 5‴ and 1⁗ (Fraga et al., 2008; Fig. 2), distinguishing it from others. *Coolia guanchica* has fewer hypothecal ornamentations but contains larger and more numerous pores, compared to *C. canariensis* and a smoother surface than *C. areolata* (David et al., 2020; Supplementary Fig. 1).

However, throughout the *C. canariensis* phylogroups, this morphological approach has been problematic. When using the 28S rRNA gene as a molecular marker, newly isolated strains and other isolates from previous studies, form four well-supported clades: phylogroup I, II, III, and IV. Phylogroup IV is a sister to phylogroup III, isolated from both Atlantic and Pacific Oceans, while phylogroup I and II have only been isolated from the Atlantic Ocean. In addition, the ITS p-distance values between strains in phylogroup IV and other phylogroups (II, III) is more than 0.04 which is often able to separate most free living dinoflagellate species (Litaker et al., 2007). The secondary structure of ITS2 also been used for delimitation of species boundaries, however, this was not performed in this study (Leaw et al., 2016). The differences in genetics and geographical location would indicate that phylogroup IV represents a new species, distinct from other known phylogroups in the *C. canariensis* clade (Jeong et al., 2012; Nascimento et al., 2019; David et al., 2020).

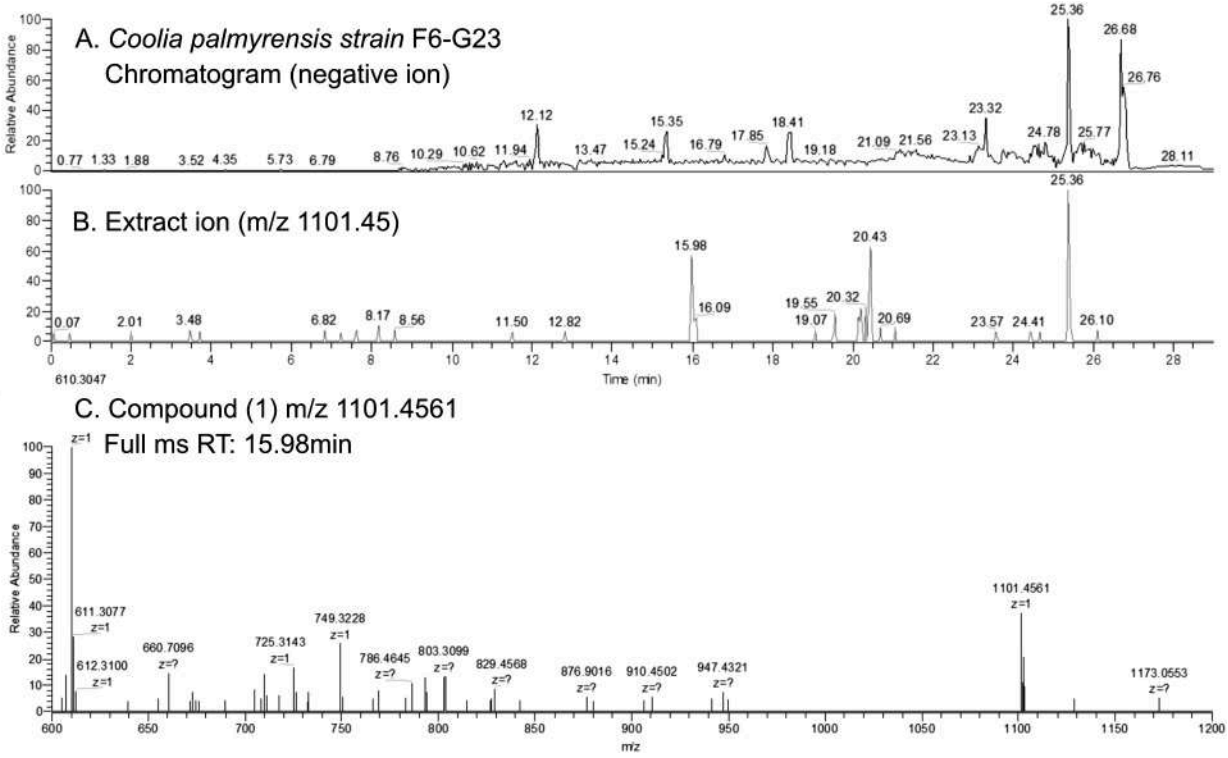

Fig. 7. NanoLCMS (negative ion) profile of water-soluble extract of *Coolia palmyrensis* strain F6-G23. A) Chromatogram; B) extract ion (*m/z* 1101.45); C) compound 1 (*m/z* 1101.4561, RT–15.98 min).

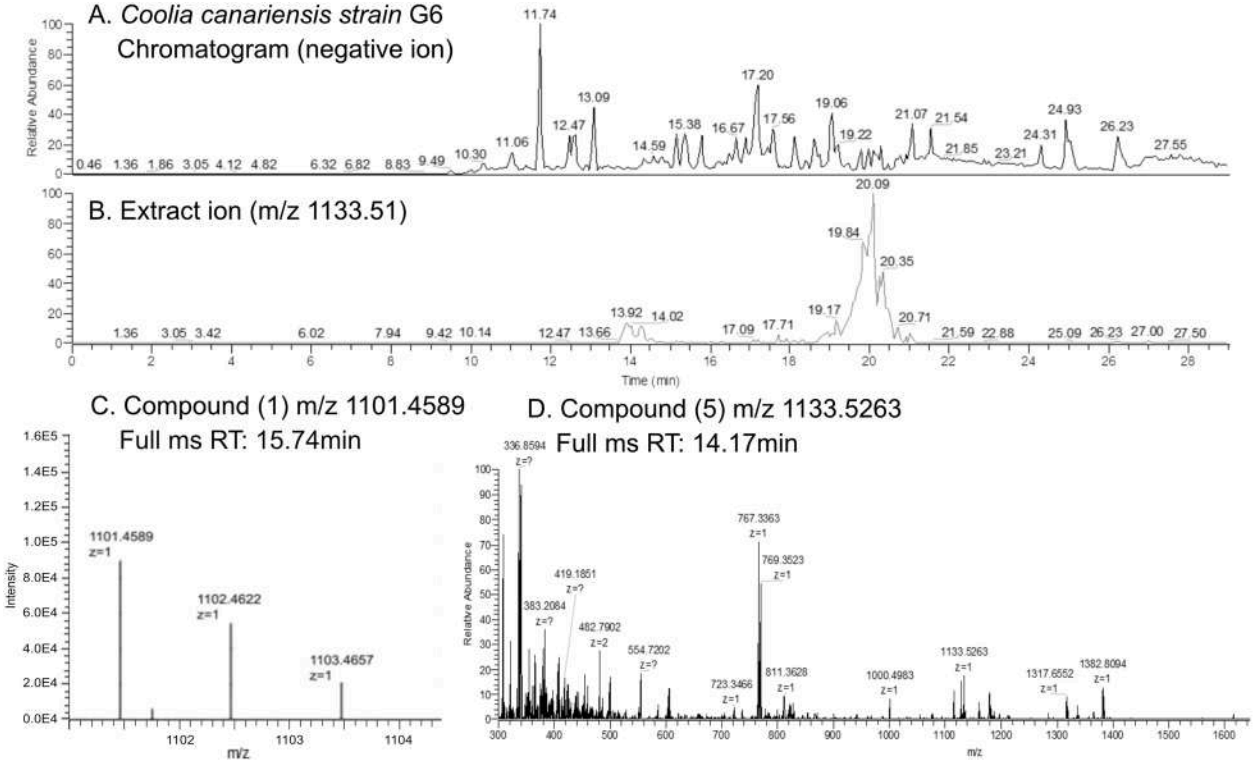

Fig. 8. NanoLCMS (negative ion) profile of water-soluble extract of *Coolia canariensis* strain G6. A) Chromatogram; B) extract ion (*m/z* 1133.51); C) compound 1 (*m/z* 1101.4589); D) compound 5 (*m/z* 1133.5263, RT–14.17 min).

4.3. Variability and diversity of toxic profiles of Coolia

The new lineage of *C. canariensis* from Guam appeared to be nontoxic to *Artemia* in our bioassays. And due to low abundance, confirmation of a YTX analogue in LCMS was not achieved. Similarly, the Guam strain of *C. canariensis* phylogroup III tested negative in both *Artemia* assay and

LCMS profile which matches with previous studies where *C. canariensis* phylogroup III appears to be a nontoxic lineage (Fraga et al., 2008; Laza-Martinez et al., 2011; Leung et al., 2017).

Both *C. malayensis* strains NIES-3637 and G10 were toxic to *Artemia* in our bioassays. This was confirmed with LCMS showing YTX analogues with characteristic MS^2 fragmentation pattern of sulphonate loss, $-SO_3$ (80 dalton). Differences in LCMS profile from strains G10 and NIES-3637 show intraspecies variation on the type and amount of toxins being produced. This result supports previous bioassay-based reports observing intraspecies variation in toxicity (Leung et al., 2017). Toxicity of each strain could be due to a mixture of different chemical compounds being produced by the dinoflagellates. Furthermore, in this study the LCMS profile did not detect several compounds (compounds **3** and **4**) from of *C. malayensis* strain identified by Wakeman et al. (2015). This indicates that the variation in toxicity of clones may be influenced by not only toxin concentration but also by the type of toxic analogues produced (Neely and Campbell, 2006).

Coolia palmyrensis strain F6-G23 tested negative in the *Artemia* bioassay showing variability compared to strains from the central Pacific and West Atlantic (Karafas et al., 2015). This may be attributed to the low abundance of the potentially toxic compounds, or variation in sensitivity of organism used in the assay (Leung et al., 2017). *Coolia tropicalis* (strain G6), tested negative in the bioassay as well as LCMS. However other strains have been shown to be toxic (Faust, 1995; Rhodes et al., 2014; Tibiriçá et al., 2020). This particular strain was unable to form a dense culture and eventually died which could have influenced toxin production, due to sub-optimal growth conditions. Abundance of detected toxins did not directly relate to the lethality of each strain. The overall lethality could be therefore influenced by other compounds such as gambierones (Tibiriçá et al., 2020) or other compounds yet to be identified.

5. Conclusion

Tropical and subtropical regions of the Pacific Ocean harbor a large biodiversity of the genus *Coolia*. In this study, four of the eight known species, *C. malayensis, C. palmyrensis, C. canariensis, C. tropicalis*, were found in Guam. A new non-toxic (exibits minimal toxic effect) phylogroup (phylogroup IV) belonging to the *Coolia canariensis* species complex was also discovered. Additional studies on the morphology, and a more comprehensive biogeographical sampling scheme would aid in deepening our understanding of this cryptic species. This study also showed that the production of toxic compounds in *Coolia* can vary in the same strain; and this variation may be influenced by differential compositions of toxic compounds being expressed. More detailed analyses will be needed to elucidate what factors contribute to the differential expression of these toxic compounds.

Author's contribution

PYH and KCW designed the project; KCW conducted field sampling; PYH and KCW isolated and establish clonal strains of *Coolia*. PYH and MCR conducted experiments and analysis; PYH, KCW, MCR, SL, FH interpreted the data, drafted and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2021.102120.

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