Harmful Algae 44 (2015) 8–19

Contents lists available at ScienceDirect

Harmful Algae

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

Morphology, phylogeny and novel chemical compounds from *Coolia malayensis* (Dinophyceae) from Okinawa, Japan

Kevin C. Wakeman [a,*], Aika Yamaguchi [b], Michael C. Roy [a], Holger Jenke-Kodama [a]

[a] Okinawa Institute of Science and Technology, 1919-1 Tancha, Onna-son, Kunigami, Okinawa 904-0495, Japan
[b] Kobe University Research Center for Inland Seas, Kobe 657-8501, Japan

ARTICLE INFO

Article history:
Received 16 August 2014
Received in revised form 25 December 2014
Accepted 22 February 2015
Available online 14 March 2015

Keywords:
Coolia malayensis
Cooliatoxin
High-resolution MS
Morphology
NanoLC/MS
Molecular phylogeny

ABSTRACT

Marine benthic dinoflagellates within the genus *Coolia* have been reported to produce natural products, some of which are known to be toxic (i.e., cooliatoxin). To date, five species of *Coolia* have been reported in tropical and temperate waters around the world; however, very few studies have combined detailed morphological and molecular data with chemical analyses. In this study, a clonal culture of *Coolia malayensis* was isolated and mass cultivated from a coral reef on the island of Okinawa, Japan. Analysis of the thecal plate morphology and molecular phylogeny from 28S rDNA strongly supported the close relationship between this new isolate of *C. malayensis* from Okinawa and other isolates of *C. malayensis* from around the world. Following methanol extraction of 250 L of mass culture, chemical analyses using NanoLiquid chromatography mass spectrometry revealed the mass profiles of water-soluble and ethyl acetate-soluble parts. High-resolution mass spectrometry derived the molecular formulas of three novel disulphated polyether analogs of yessotoxin ($C_{56}H_{78}O_{18}S_2$ 1102.4 (Compound **1**), $C_{57}H_{80}O_{18}S_2$ 1116.4 (Compound **2**), and $C_{57}H_{78}O_{19}S_2$ 1130.4 (Compound **3**)); two potential homologous compounds (Compounds **4** and **5**) were also observed on the high-resolution mass, albeit with low signal intensity. The five compounds in the *C. malayensis* from Okinawa are composed of less oxygen, compared to cooliatoxin and other analogs of yessotoxin, suggesting the metabolites produced by *C. malayensis* are unique to those previously reported from other strains of *Coolia*.

1. Introduction

Coolia is a genus of benthic marine dinoflagellate with broad distributions in tropical and temperate regions. Currently, five species of *Coolia* have been established from various locations including Belgium (Meunier, 1919), Malaysia (Leaw et al., 2010), the Canary Islands (Fraga et al., 2008), Belize (Faust, 1995) and the South West Indian Ocean (Ten-Hage et al., 2000). Like other benthic dinoflagellates, *Coolia* produces natural products, some of which have been shown to be toxic (Holmes et al., 1995; Nakajima et al., 1981; Rhodes and Thomas, 1997; Rhodes et al., 2010; Yasumoto et al., 1980, 1987). This has motivated research seeking to better understand the toxicity, distributions, biodiversity and ecology of *Coolia* in other areas like New Zealand (Rhodes et al., 2014), the Great Barrier Reef (Australia) (Momigliano et al., 2013), the South Pacific Ocean (Rhodes et al., 2010), Japan (Fukuyo, 1981; Nakajima et al., 1981; Yasumoto et al., 1980, 1987), Korea (Jeong et al., 2012; Shah et al., 2014), Spain (Laza-Martinez et al., 2011), and at sites around the Mediterranean (Aligizaki and Nikolaidis, 2006; Armi et al., 2010; Dolapsakis et al., 2006; Pagliara and Caroppo, 2012; Zingone et al., 2006) and Egypt (Ismael, 2014).

Holmes et al. (1995) reported the first toxin (cooliatoxin) isolated from a strain of *Coolia*: a mono-sulphated polyether (M = 1062) similar to yessotoxin; other work has also isolated and characterized novel natural products from cultures of *Coolia* (Akasaka et al., 2000; Liang et al., 2009; Tanaka et al., 1998). Since cooliatoxin was first reported, only some strains of *Coolia* (*C. tropicalis* and *C. malayensis*) have been shown to produce toxins in biological assays on mice, fish, invertebrates or hemolytic assays (Holmes et al., 1995; Laza-Martinez et al., 2011; Mohammad-Noor et al., 2004; Nakajima et al., 1981; Penna et al., 2005; Rhodes and Thomas, 1997; Rhodes et al., 2010, 2014; Ten-Hage et al., 2000; Yasumoto et al., 1980, 1987). More recently, some work has looked at *Coolia* as a source of bioactive compounds (Shah et al., 2014).

Still, the diversity, distribution and chemical production in this genus is not well understood. It is likely that many more species and their chemical characteristics have yet to be described (Jeong

* Corresponding author. Tel.: +81 098 966 8481; fax: +81 098 966 2890.
E-mail address: wakeman.kevin@gmail.com (K.C. Wakeman).

http://dx.doi.org/10.1016/j.hal.2015.02.009
1568-9883/

et al., 2012; Mohammad-Noor et al., 2004; Momigliano et al., 2013; Shah et al., 2014). Much of the recent work on *Coolia* has emphasized morphology and/or molecular data (Faust, 1992, 1995; Fukuyo, 1981; Jeong et al., 2012; Leaw et al., 2010; Mohammad-Noor et al., 2013; Ten-Hage et al., 2000), or chemical analyses and screening for toxins (Holmes et al., 1995; Laza-Martinez et al., 2011; Mohammad-Noor et al., 2004; Nakajima et al., 1981; Rhodes and Thomas, 1997; Yasumoto et al., 1980, 1987), with the exception of a handful that have combined taxonomy, phylogenetics and chemical analyses (Fraga et al., 2008; Penna et al., 2005; Rhodes et al., 2010, 2014). Within the context of species diversity, distribution and natural product chemistry, merging detailed morphological data with chemical analyses will help us better understand *Coolia*.

To this end, we analyzed a strain of *Coolia malayensis* isolated from the Southwest Pacific Ocean on the island of Okinawa, Japan. The aim of this work was to (1) give a detailed morphological and phylogenetic report of *C. malayensis* from Okinawa, Japan, and (2) use mass cultivation and chemical analyses, such as high-pressure liquid chromatography–mass spectrometry (HPLC–MS), to tease apart the diversity of chemical and potentially bioactive compounds found in this benthic dinoflagellate.

2. Materials and methods

2.1. Collection and culturing of Coolia malayensis

Small amounts of turf algae were collected in June 2013 from a shallow reef near Teniya, Okinawa, Japan 26°53′51.67″ N 128°08′28.06″ W. Samples were stored in plastic containers in sea water, transported to the lab and observed with an inverted microscope (Olympus CKX41, Olympus Corporation, Tokyo, Japan). Single cells were isolated using hand-drawn glass pipettes. Cells were washed twice (until clean) in filtered, autoclaved seawater, and one cell was used to establish cultures in 24-well tissue culture plates containing Daigo's IMK culture medium (Wako, Osaka, Japan). Culture plates were covered with parafilm and placed at 25 °C, under a 12 h:12 h light/dark cycle (150 photons/m^2/s).

Mass culturing of a clonal culture was performed by filter-sterilizing 2 L of seawater through a 0.22 μm filter. The filtered seawater was then placed in a 3 L flat-bottom, glass flask (Sibata Scientific Technology, Japan). Daigo's IMK culture medium was added to each flask, following manufacturer protocols. Each flask was autoclaved at 121 °C for 25 min, and left to cool at room temperature for 24 h. Flasks were inoculated with 1 ml of dense culture, and placed at 24 °C, under a 12 h:12 h light/dark cycle (150 photons/m^2/s) for 3 weeks. The strain of *C. malayensis* used in this study was deposited in the National Institute for Environmental Studies (NIES), Japan (NIES-3637).

2.2. Light and confocal microscopy

Differential interference contrast (DIC) images of *Coolia malayensis* were taken with an Olympus BX51TF (Olympus Corporation, Tokyo, Japan), connected to a Nikon DS-L3 color digital camera (Nikon Corporation, Tokyo, Japan). To elucidate thecal plate morphology, cells were stained with calcofluor [0.5 mg/ml] (1:1 mixture of calcofluor and culture medium) and mounted on glass slides. Stacked images were acquired using an Olympus FV10i (Olympus, Tokyo, Japan).

2.3. Scanning electron microscopy

Round coverslips were coated with poly-L-lysine and left to dry, while covered at room temperature. Treated coverslips were placed at the bottom of a 24-well tissue culture plate. Approximately 10 ml of culture was fixed with 30 μl of Lugol's Fixative in 15 ml falcon tubes for two days at room temperature. Cells were isolated from the fixative solution using hand-drawn glass pipettes and placed on the poly-L-lysine-coated coverslip. The coverslip containing the cells was washed two times in distilled water, and dehydrated through a graded series of ethanol (70%, 85%, 90%, 95%, 100%, 100%, 100%) for 5 min at each step. The coverslip was then submerged in a 1:1 mixture of 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and ethanol for 10 min, followed by three washes with 100% HMDS lasting 15 min each. The coverslip was left to air-dry for 5 min, and then placed in a 60 °C oven for 5 min, to ensure complete evaporation of the HMDS. Coverslips were mounted on aluminum stubs, sputter-coated for 4 min with gold (Varian Turbo-V 70, Japan) and viewed with a Helios NanoLab 650 (FEI Company, USA) scanning electron microscope.

2.4. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from a frozen pellet of *Coolia malayensis* (500 μl of culture spun at 10,000 × *g* for 10 s) using the DNeasy Blood and Tissue Kit (Qiagen, Japan), following manufacturer protocols. Outside primers, SR1 and LSU R2, as well as an internal primer, 25 F1, was used in an initial 25 μl PCR with EconoTaq 2× Master Mix (Lucigen Corp., USA), using the following program on a thermocycler: Initial denaturation at 94 °C for 2:00 min; 35 cycles of denature at 94 °C for 0:30 s, anneal at 48 °C for 0:30 s, extension at 72 °C for 3:50 min, final extension 72 °C 9:00 min. The PCR product was diluted (1/100) in distilled water, and 1 μl was used in subsequent 25 μl PCRs in EconoTaq 2× Master Mix to amplify LSU rDNA and the ITS region of the rDNA. Amplification of LSU rDNA was performed with primer pairs, LSUD3A – LSUR2 and 25F1 – 25R1. The following protocol was used on a thermocycler: Initial denaturation at 94 °C for 2:00 min; 25 cycles of denature at 94 °C for 0:30 s, anneal at 55 °C for 0:30 s, extension at 72 °C for 1:20 min, final extension 72 °C 9:00 min. In the case of 25F1 and 25R1, an annealing temperature of 48 °C was used. A 750 bp region of the ITS region was amplified with primers, Lp1F1 and 25F1R (Howard et al., 2009; Takano and Horiguchi, 2006), using the following thermocycler program: Initial denaturation at 94 °C for 2:00 min; 25 cycles of denature at 94 °C for 0:30 s, anneal at 48 °C for 0:30 s, extension at 72 °C for 1:20 min, final extension 72 °C 9:00 min. Primer sequences are specified in Table 1.

Table 1
Primer sequences used in this study.

Primer name	Sequence	Direction	Reference
SR1	5′-TACCTGGTTGATCCTGCCAG-3′	F	Nakayama et al. (1996)
25F1	5′-CCGCTGAATTTAAGCATAT-3′	F	Kogame et al. (1999)
LSUD3A	5′-GACCCGTCTTGAAACACGGA-3′	F	Nunn et al. (1996)
25R1	5′-CTTGGTCCGTGTTTCAAGAC-3′	R	Kogame et al. (1999)
LSUR2	5′-ATTCGGCAGGTGAGTTGTTAC-3′	R	Takano and Horiguchi (2006)
Lp1F1	5′-GTCCCTGCCCTTTGTACAC-3′	F	Howard et al. (2009)
25F1R	5′-ATATGCTTAAATTCAGCGG-3′	R	Takano and Horiguchi (2006)

PCR products were checked for size on a 2% agarose gel, and cloned into a pCR 2.1 vector using a Topo TA cloning kit (Invitrogen, Tokyo, Japan). Four viable colonies from each reaction were picked, PCR-screened using vector primers, and sequenced by Fasmac (Tokyo, Japan). Sequences were initially checked using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) and deposited in GenBank (KJ933432-KJ933439).

2.5. Phylogenetic analyses

Two alignments were constructed for this study: (1) a 45-taxon alignment (287 unambiguously aligned sites) containing the 28S rDNA sequences from the *Coolia malayensis* isolated in this study and *Prorocentrum micans* (outgroup) and (2) a 27-taxon alignment (278 unambiguously aligned sites) containing the ITS1, 5.8S rDNA and ITS2 regions of *C. malayensis* from this study and the comprehensive diversity of ITS regions from *Coolia* spp. available in GenBank. The 28S rDNA alignment was initially constructed using MUSCLE (Edgar 2004) and was edited and fine-tuned using Mesquite (Maddison et al., 2007); gaps and ambiguously aligned regions were excluded from the analyses. The ITS regions were initially aligned using ClustalW (Larkin et al., 2007) and fine-tuned with Mesquite. MEGA 5 (Tamura et al., 2011) was used to calculate percent differences between the ITS rDNA of *Coolia*. The alignments used in this study are available from the authors by request.

Jmodeltest 2.1.4 selected a TIM1 + I model of evolution under Akaike Information Criterion (AIC) and AIC with correction (AICc) (proportion of invariable sites = 0.3370) (Posada and Crandall 1998). Garli0.951-GUI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) was used to infer a maximum likelihood (ML) tree and for ML bootstrap analyses (1,000 pseudoreplicates, one heuristic search per pseudoreplicate) (Zwickl 2006).

Bayesian posterior probabilities were calculated using the following parameters on the program MrBayes v3.2.2. (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). A GTR

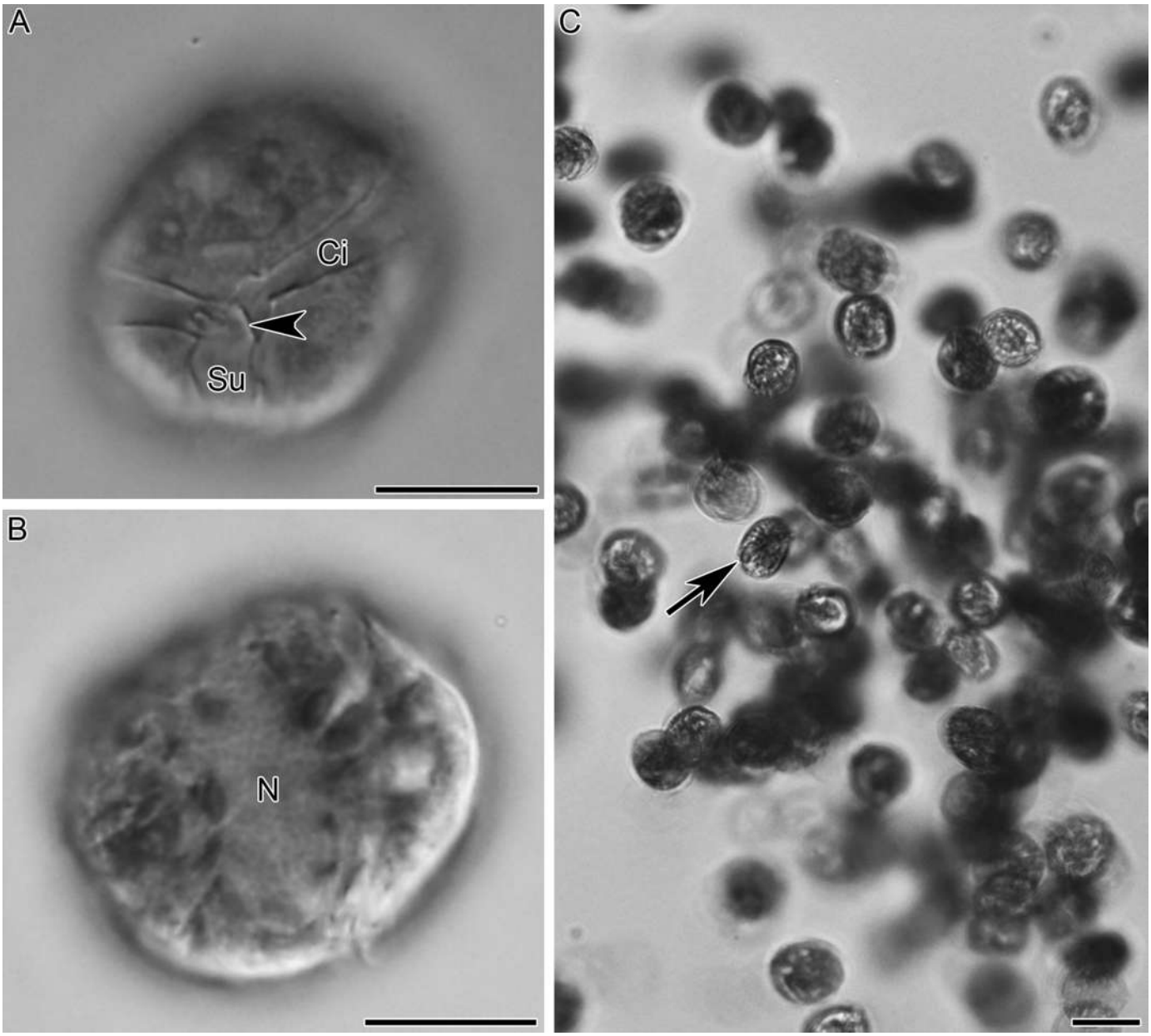

Fig. 1. Differential interference contrast (DIC) micrographs of *Coolia malayensis*. (A and B) Light micrographs of an individual cell showing the cingulum (Ci), sulcus (Su), flagellum (arrowhead) and nucleus (N). (C) Low-magnification micrograph showing swimming cells in culture (arrow). Scale bars: A, B = 15 μm; C = 50 μm.

model with a gamma-distributed rate variation across sites and a proportion of invariable sites was used as the evolutionary model. The program was set to operate four Monte-Carlo-Markov Chains (MCMC) starting from a random tree. A total of 1,000,000 generations was calculated with trees sampled every 100 generations. The first 2,500 trees in each run were discarded as burn-in. Burn-in was confirmed manually, and majority-rule consensus trees were constructed; posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees.

2.6. Methanol extract preparation of Coolia malayensis

Cultures were filtered through glass fiber filters (150 mm, 0.8 μm particle retention, GA-200, Advantec MFS, Japan). Approximately 16 L of culture was passed through one filter; a total of 250 L was cultivated during this study. Filters containing the biomass were stored in falcon tubes at −80 °C until ample material had been collected (77.6 g, wet weight). The frozen biomass was thawed briefly (15 min) at room temperature and transferred to a 3 L Erlenmeyer Flask. Methanol (1.5 L) was added and stirred to break apart the glass fiber filters using a long metal spatula. The suspension was sonicated (5 min) and left at room temperature for 30 min with occasional stirring. The methanol extract was filtered by vacuum filtration through a glass fiber filter. The methanol extraction was repeated two more times. The last extraction was carried out over night at room temperature. The pooled methanol extracts (4.5 L) were evaporated using a rotary evaporator (Büchi Rotavapor R-215, Switzerland) that resulted in a viscous crude extract. The crude extract was suspended in methanol-distilled water (1:1, 120 ml) and sonicated until dissolved. Finally, the methanol–water crude extract was defatted using ethyl acetate (150 ml, three times) in a separatory funnel (500 ml, Sibata scientific technologies, Japan). The water-soluble and ethyl acetate-soluble parts were concentrated using the rotary evaporator. The concentrated extracts (each 50 ml) were fully dried in a vacuum concentrator (40 °C) and stored at −30 °C before mass analysis and fractionation.

2.7. Nanoliquid chromatography mass spectrometry

A Thermo Scientific Hydride Mass Spectrometer (LTQ Orbitrap) was used for mass spectrometry (MS) data collection. The mass spectrometer was equipped with high-pressure liquid chromatography (HPLC) (Paradigm MS4, Michrom Bioresources Inc.), an autosampler (HTC PAL, CTC Analytics) and a nanoelectrospray ion source (NSI). Three tandem spectra were generated for each targeted compound in negative ion mode. MS^1 spectrum was acquired at 60,000 resolution in FTMS mode (Orbitrap); full mass range m/z 300–2000 Da, with capillary temperature (200 °C) and spray voltage (1.9 kV). MS^2 spectrum was configured as data-dependent manner to select top ions from the MS^1 spectrum, using ITMS mode, with optimized collision energy (35%) and isolation window (2 Da). For MS^3 spectrum, a neutral loss-dependent mode was used to monitor the sulphonate loss ($-SO_3$, 80 Da) from the precursor ion detected in the MS^2 scan and automatically triggered data-dependent MS^3 fragmentation of the neutral loss precursor

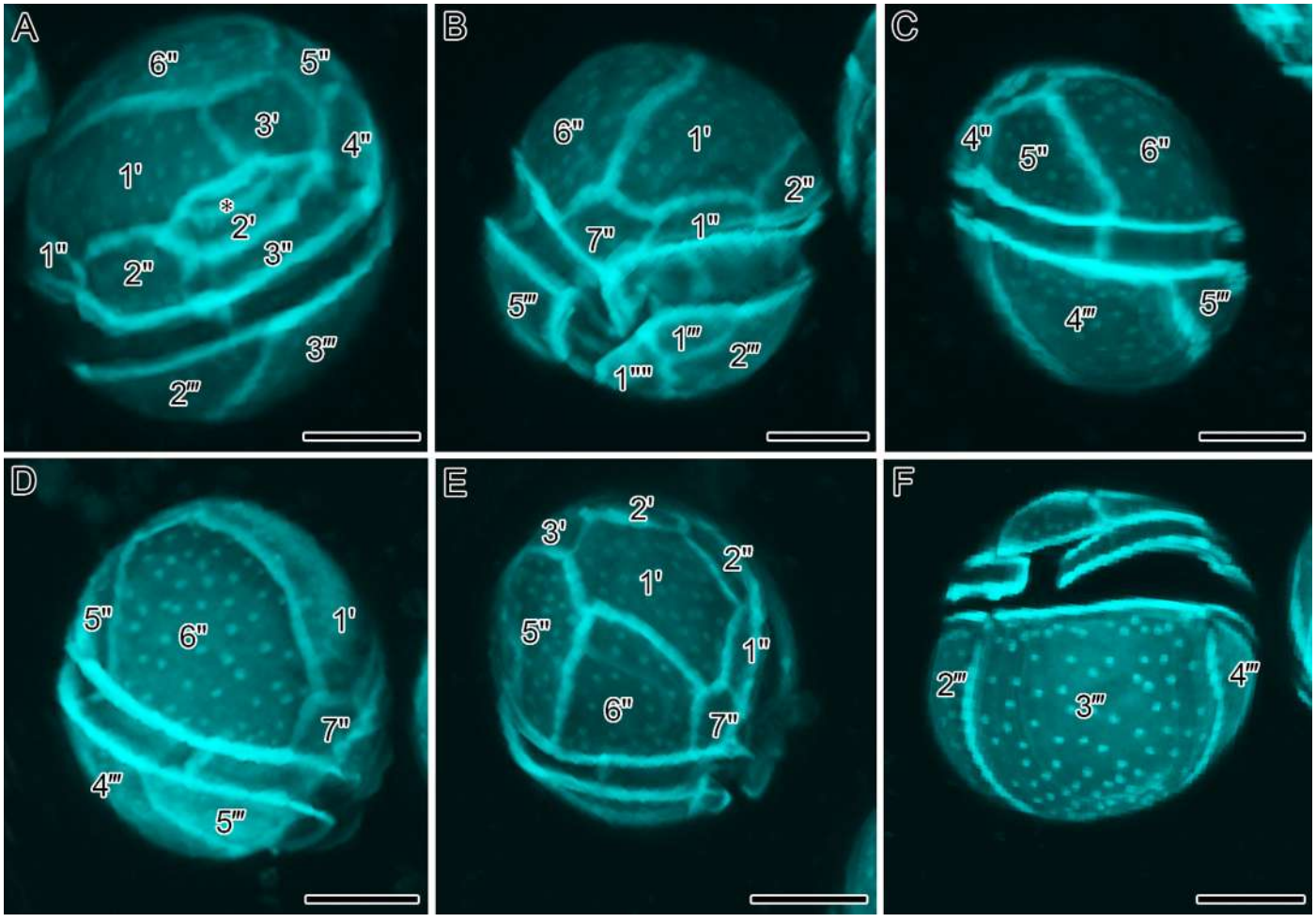

Fig. 2. Confocal images of calcofluor-stained *Coolia malayensis*. (A–F) Images showing the thecal pattern of the epitheca and hypotheca. Scale bars: A–F = 10 μm.

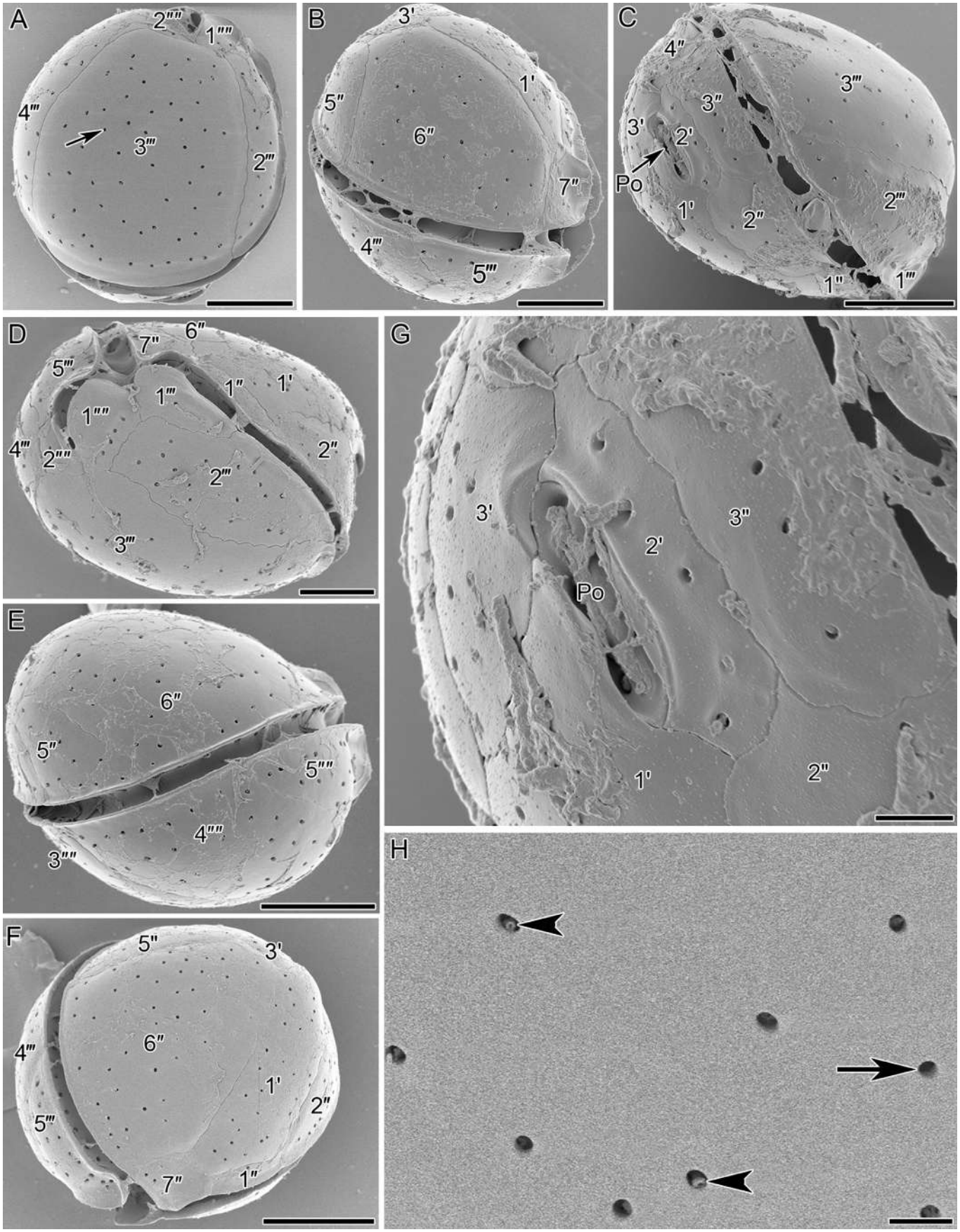

Fig. 3. Scanning electron microscope (SEM) images showing the general morphology and thecal pattern of *Coolia malayensis*. (A–F) SEM images showing the thecal plate pattern, as well as pores in the thecal plates (arrow) and the apical pore (Po). (G) High-magnification SEM image showing the apical pore (Po) and the thecal pattern near the pore. (H) High-magnification SEM showing pores with internal perforations (arrowhead) and those without (arrow). Scale bars: A–C, E, F = 10 μm; D = 5 μm; G, H = 1 μm.

ion. Clean samples were separated on a capillary reverse phase column (50 mm × 0.15 mm, 3 μm, C_{18}, Vydac). A 24-min step-gradient (5% B for 0.0–2.0 min, 5–30% B for 2.0–15.0 min, 30–90% B for 15.0–20.0 min, hold 90% B until 24.0 min, equilibration 5% B from 24.0–30.0 min; where solvent A is aqueous-acetonitrile 98:2 and solvent B is aqueous-acetonitrile 2:98, both containing 0.1% formic acid, flow rate 2.0 μl/min, 1 injection 2 μl) was used for the metabolite separation.

2.8. Crude extract desalting and partial fractionation for NanoLC–MS analysis

Samples were desalted using a ZipTip C_{18}/P10 (Millipore) and HLB solid phase extraction columns (1 ml, Oasis, Waters), prior to NanoLC–MS analysis. Before desalting, the ZipTip and HLB columns were activated with methanol and aqueous–methanol–formic acid (5:5:0.02), and finally equilibrated with aqueous–methanol–formic acid (9:1:0.05). The ZipTip C_{18} and HLB recovered the majority (~99%) of metabolites.

To clear the solution, an aliquot of the aqueous-soluble part was reconstituted (100 mg, 0.5 ml, aqueous–methanol–formic acid 9:1:0.05) and centrifuged (14,000 × *g*, 10 °C, 10 min). A portion of the clear solution (20 μl) was desalted using a ZipTip C_{18}. Retained material was eluted with aqueous-methanol-formic acid (5:5:0.02, 4 μl) in a sample vial. The remaining portion was passed over a HLB column (1 ml) and desalted by washing with aqueous–methanol–formic acid (9:1:0.05, 3 ml). The HLB-retained organic materials were eluted with methanol (1 ml) and dried to a powder (6 mg). This methanol-eluted part was subjected to further fractionation to get homogenous material. In a similar fashion, the lipophilic extract (ethyl acetate part) was suspended in aqueous–methanol–formic acid (7:3:0.05, 0.5 ml) and centrifuged (14,000 × *g*, 10 °C, 10 min), resulting in a clear solution. The clear solution was collected and insoluble (non-polar) materials were discarded. After dilution (100 μg/ml) with the washing solvent, all samples (eluted from the ZipTip and HLB, and the polar part of the lipophilic extract) were analyzed by NanoLC–MS.

The methanol-eluted materials from HLB column was fractionated over an ODS column (DSC-18, 50 mg, 1 ml, Supelco) to give three fractions: (a) aqueous–methanol–formic acid (9:1:0.05), (b) aqueous–methanol–formic acid (5:5:0.02), and (c) methanol–formic acid (100:0.2). Target molecules were concentrated in fraction (b). Further, HPLC (Nexera LC-30AD, Diod Array Detector SPD-M20A, Autosampler SIL-30AC, Shimadzu) separation of fraction (b) on an ODS column (Cosmosil 5C18-MS-II, 250 mm × 4.6 mm; solvent A: aqueous–methanol 95:5 and solvent B: methanol, both containing 0.1% formic acid; gradient: 15–90% B for 0.0–10 min, hold 90% B until 13.0 min, equilibration 15% B from 13.0–15.0 min, flow rate 1 ml/min) and fraction collection resulted in 10 sub-fractions (1.10 min/fraction, collection 2.0–13.0 min). The target molecules were found in sub-fraction six. This partially homogenous sub-fraction was used for details MS data collection.

3. Results and discussion

3.1. Morphology Coolia malayensis from Okinawa

Cells of *Coolia malayensis* were roughly spherical with an average length of 27 μm (range = 20–32 μm, *n* = 60) and width of 28 μm (range = 22–33 μm, *n* = 60) (Fig. 1A–C). The kidney-shaped nucleus was located in the center of the cell (Fig. 1B). The thecal plate arrangement (Po, 3′, 7″, 6c,?s, 5‴, 2⁗) was inferred from light micrographs of calcofluor-stained cells (Fig. 2A–F) and SEM images (Fig. 3A–H).

Past work on *Coolia* has emphasized thecal plate morphology as diagnostic for species-level identification of *C. malayensis* (Leaw et al., 2010; Jeong et al., 2012). This includes the relative size of the hypothecal 3‴ plate compared to the neighboring 4‴ plate, the shape of the 3′ plate on the epitheca, and the ratio of width to length of the epithecal 7″ plate (Leaw et al., 2010; Jeong et al., 2012). With regard to our isolate from Okinawa, the 3‴ plate is larger than the 4‴ plate (Fig. 2F; Fig. 3A), and the shape of the 3′ plate on the epitheca was pentagonal (Fig. 2A); these characteristics are similar to Korean and Malaysian strains of *C. malayensis* (Leaw et al., 2010; Jeong et al., 2012). Moreover, the ratio of width to length of the 7″ plate in the strain from Okinawa was 1.4 (Figs. 2B, D and 3F). These findings led to the conclusion that this isolate was morphologically similar to *C. malayensis* reported from Malaysian and Korean waters. High-magnification SEM images of our isolate of *C. malayensis* also revealed an apical pore complex and perforated pores in the thecal plates (Fig. 3G and H). However, it has been suggested that culture conditions might introduce variability in thecal plate morphology and cell shape in *Coolia* (Jeong et al., 2012). Therefore, molecular data will be useful as more species are reported and cultivated.

3.2. Molecular phylogenetic analyses

Five distinct clades and one lineage were recovered in our analyses of the 28S rDNA from the *Coolia* spp. (Fig. 4). The 28S rDNA from the single, clonal culture of *C. malayensis* in this study grouped with high-support to other *C. malayensis* from around the world. *C. monotis* formed a single, well-supported sister clade to *C. malayensis*. Four other groups were identified in the tree: *C. canariensis* I (AM902737-AM902738), *C. canariensis* II, *C. tropicalis* and *C.* sp. from the Great Barrier Reef (HQ897277) (Fig. 4). Pairwise comparison of the aligned ITS1, 5.8S rDNA and ITS2 regions showed that intraspecific variation between isolates of *C. malayensis* ranged from 0.0% to 0.018%. Interspecific specific variability between *C. malayensis* and the other *Coolia* spp. ranged from 0.161% to 0.165% (Supplementary Table 1).

Supplementary Table 1 related to this article can be found, in the online version, at doi:10.1016/j.hal.2015.02.009.

3.3. Mass profile and molecular identification of C. malayensis by high-resolution MS

The NanoLC–MS (negative ion) showed different molecular profiles (*m*/*z* 300–2000 Da) for the water-soluble and ethyl acetate-soluble (polar) parts, as shown in Fig. 5. Highly polar metabolites were concentrated in the aqueous part, and the most abundant molecules belonged to peptide and polypeptide classes. Searching for molecular ions that lost a sulphonate group (–SO_3, 80 Da) in the MS^2 scan revealed several molecular species resembling yessotoxin analogs (Satake et al., 1997, 1999) and cooliatoxin (Holmes et al., 1995).

A major signal was observed at *m*/*z* 1101.46 in the crude water-soluble part (Fig. 6, t_R 11.93 min). Desalting (HLB column) followed by ODS (DSC-18) fractionation and HPLC separation (as described in the experimental section) resulted in a partially homogenous fraction that contained other molecular ions (*m*/*z* 1115.47 and 1129.45), along with the major signal showing –SO_3 in the MS^2 scan. Two related minor analogs were also detected at *m*/*z* 1119.49 and 1133.51 in this fraction (Supplementary Fig. S1).

Supplementary Fig. S1 related to this article can be found, in the online version, at doi:10.1016/j.hal.2015.02.009.

High-resolution mass (HRMS) was used to infer the molecular formulas as $C_{56}H_{77}O_{18}S_2$ 1101.4589 $[M-H]^-$ Δ 4.28 mmu (Compound **1**), $C_{57}H_{79}O_{18}S_2$ 1115.4745 $[M-H]^-$ Δ 4.26 mmu (Compound **2**), and $C_{57}H_{77}O_{19}S_2$ 1129.4541 $[M-H]^-$ Δ 4.60 mmu

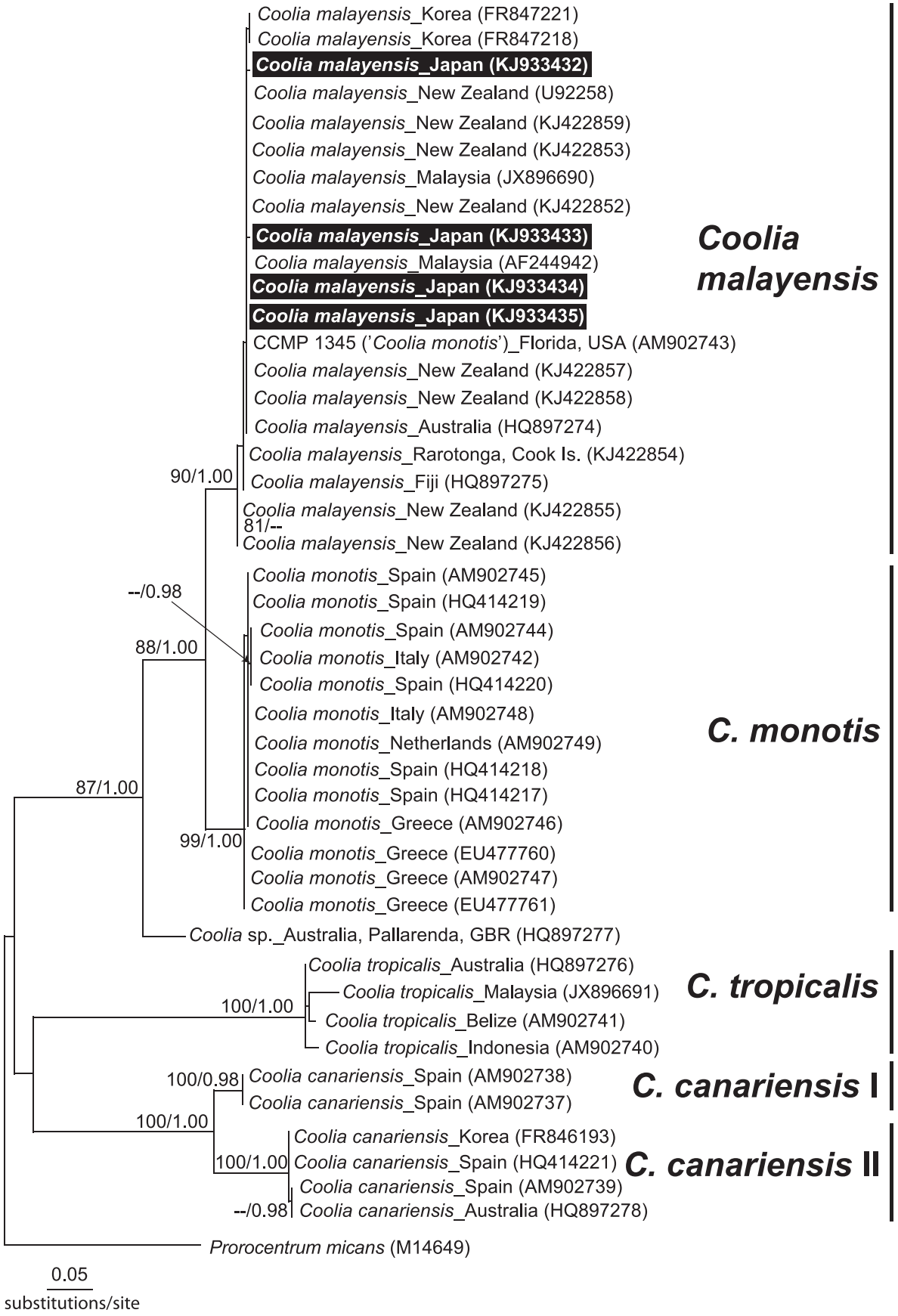

Fig. 4. Maximum likelihood (ML) tree derived from phylogenetic analyses of the 45-taxon dataset (287 unambiguously aligned sites) of 28S rDNA sequences. This tree was inferred using the TIM1 + I substitution model (−lnL = 1539.14930, proportion of invariable sites = 0.0.3370). Bootstrap support values and Bayesian posterior probabilities lower than 80 and 0.95, respectively, were not added to the tree. The four sequences from the clonal culture of *Coolia malayensis* (NIES-3637) are in bold, highlighted with black boxes.

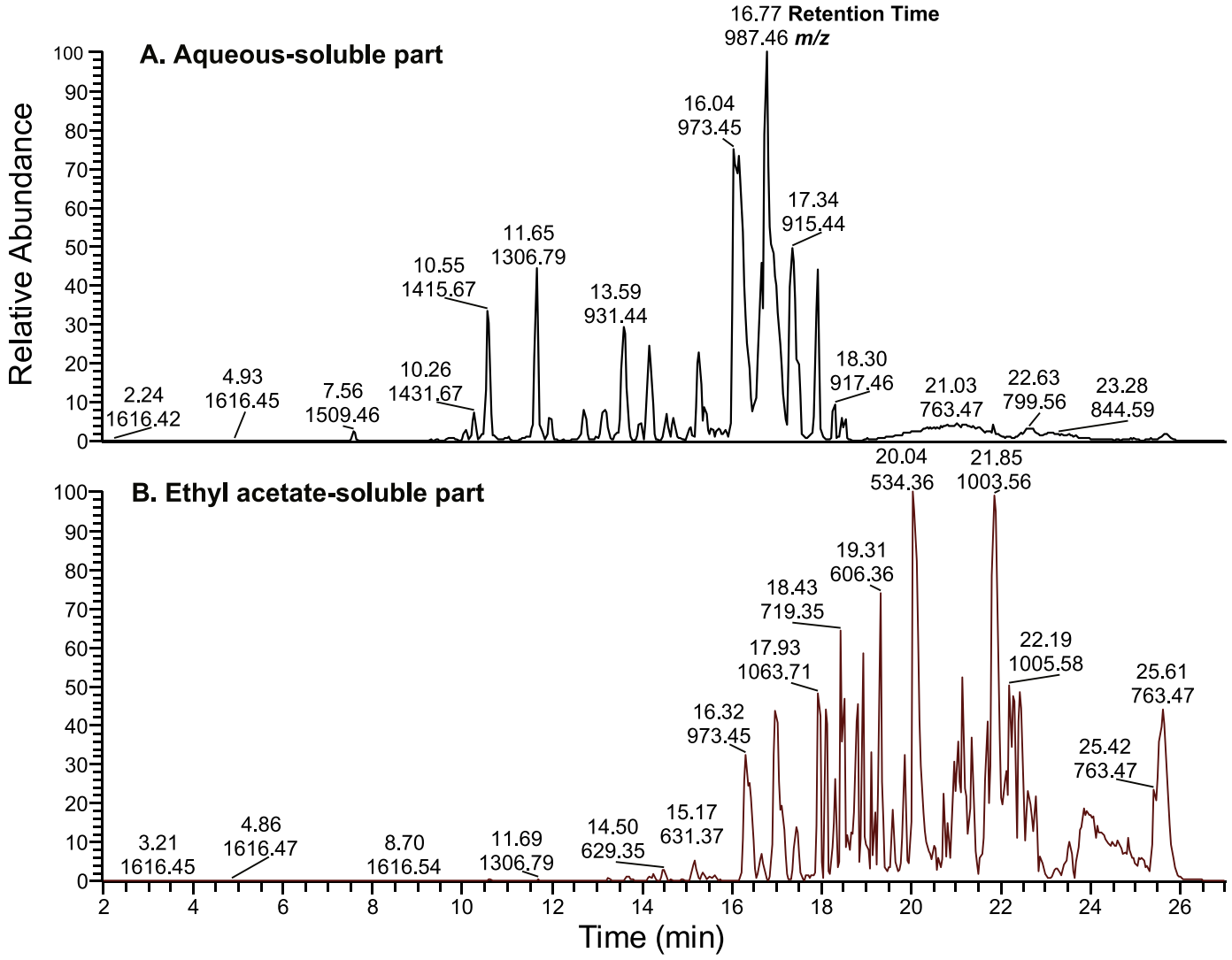

Fig. 5. NanoLC–MS (negative ion) profile of the methanol extract of *Coolia malayensis*. (A) Aqueous-soluble profile; (B) ethyl acetate-soluble profile.

(Compound **3**). All three molecules were inferred to be disulphated polyether analogs of yessotoxin. Compound **2** differed from Compound **1** only by a single methylene (–CH_2–); Compound **3** was an oxo-derivative of Compound **2**. The HRMS also suggested that other minor signals at *m/z* 1119.49 (Compound **4**) and 1133.51 (Compound **5**) could be homologues of Compounds **1**–**3**. It should be noted that Compounds **4** and **5** were not selected for MS^2 fragmentation due to their very low signal intensity.

A series of molecular ion species observed at *m/z* 573.22 $[M+2Na-2H]^{2-}$, 550.22 $[M-2H]^{2-}$, and 540.73 $[M-H_2O+2H]^{2-}$in the negative ion mode supported the molecular formula for the major compound (Compound **1**). It was further supported by the positive ions at *m/z* 1125.45 $[M+Na+H]^+$, 1120.49 $[M+NH_4]^+$, 1102.48 $[M-H_2O+NH_4]^+$, 1085.46 $[M-H_2O+H]^+$, 551.74 $[M-H_2O+NH_4+H]^{2+}$, 543.23 $[M-H_2O+2H]^{2+}$, and 534.22 $[M-2H_2O+2H]^{2+}$ (Supplementary Fig. S2). In case of Compounds **2** and **3**, only the $[M+2Na-2H]^{2-}$ ions were observed at *m/z* 580.23 and 587.22, respectively. On the other hand, both single $[M-H]^-$ and double $[M-2H]^{2-}$ charged ions were observed for Compounds **4** and **5**, similar to Compound **1**; however, their sodium adduct ions were not detected (Table 2). As shown in Fig. 7, ions related to the sulphonate loss $[M-SO_3-H]^-$ at *m/z* 1021.5, 1035.4, and 1049.4 were observed for Compounds **1**–**3**, respectively. In the MS^3 stage, the daughter ions of Compound **1** lost water, resulting in $[M-SO_3-H_2O-H]^-$ at *m/z* 1003 and other major fragments at *m/z* 877, 721, and 703 (Fig. 7). A similar mass

Table 2
High-resolution mass ion (negative) species observed for compounds **1–5** from *Coolia malayensis*.

Compound	Formula	$[M-H]^-$	$[M-2H]^{2-}$	$[M+2Na-2H]^{2-}$	$[M-H_2O+2H]^{2-}$
1	$C_{56}H_{78}O_{18}S_2$	1101.4589	550.2268	573.2288	540.7303
2	$C_{57}H_{80}O_{18}S_2$	1115.4745	–[a]	580.2371	–
3	$C_{57}H_{78}O_{19}S_2$	1129.4541	–	587.2270	–
4	$C_{57}H_{84}O_{18}S_2$	1119.4962	559.2452	–	–
5	$C_{58}H_{86}O_{18}S_2$	1133.5121	566.2532	–	–

[a] Signal was not observed.

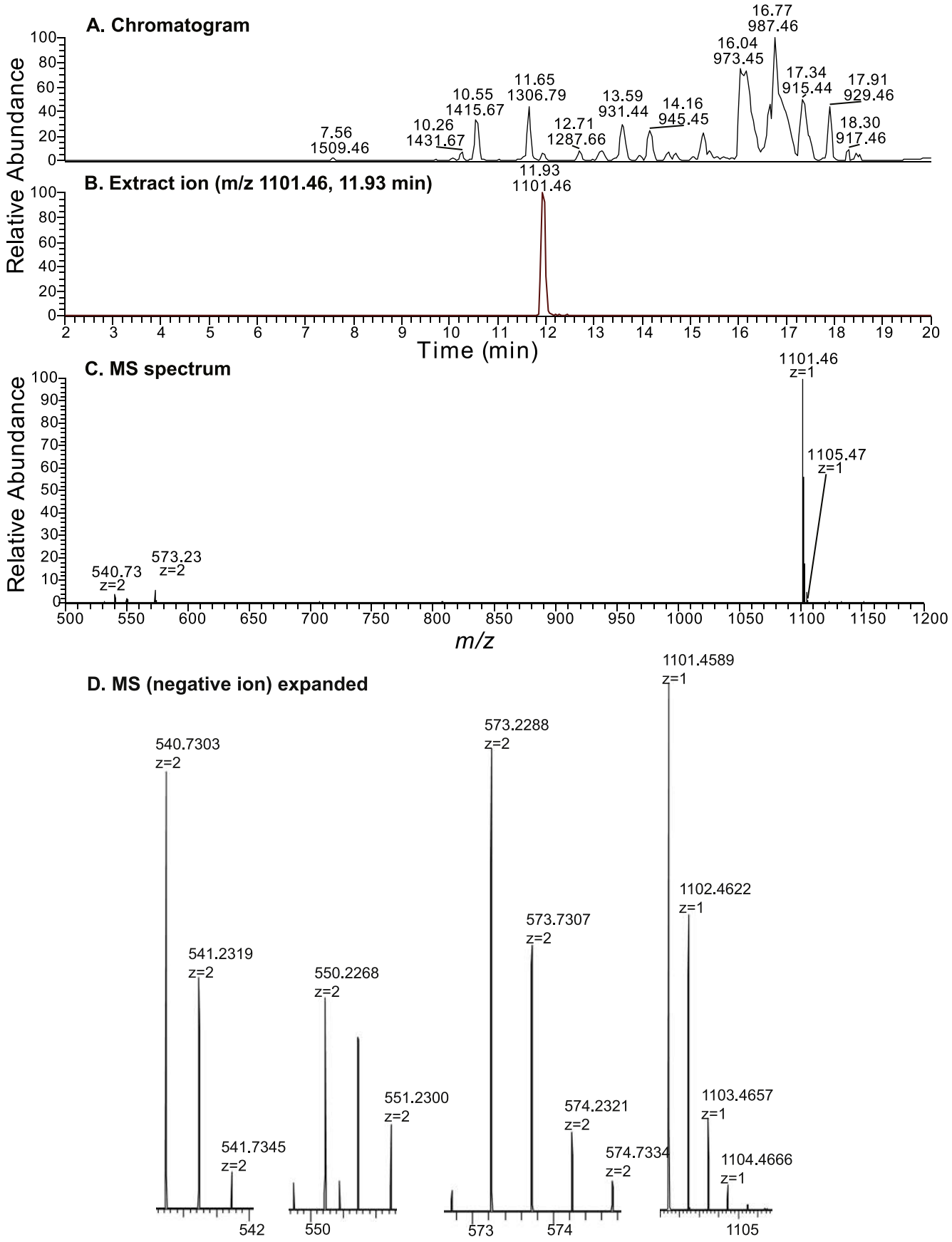

Fig. 6. MS (negative ion) of the major molecule in the methanol extract of *Coolia malayensis*. (A) Chromatogram; (B) extract ion (*m*/*z* 1101.46, 11.93 min); (C) MS spectrum; (D) MS (negative ion) expanded.

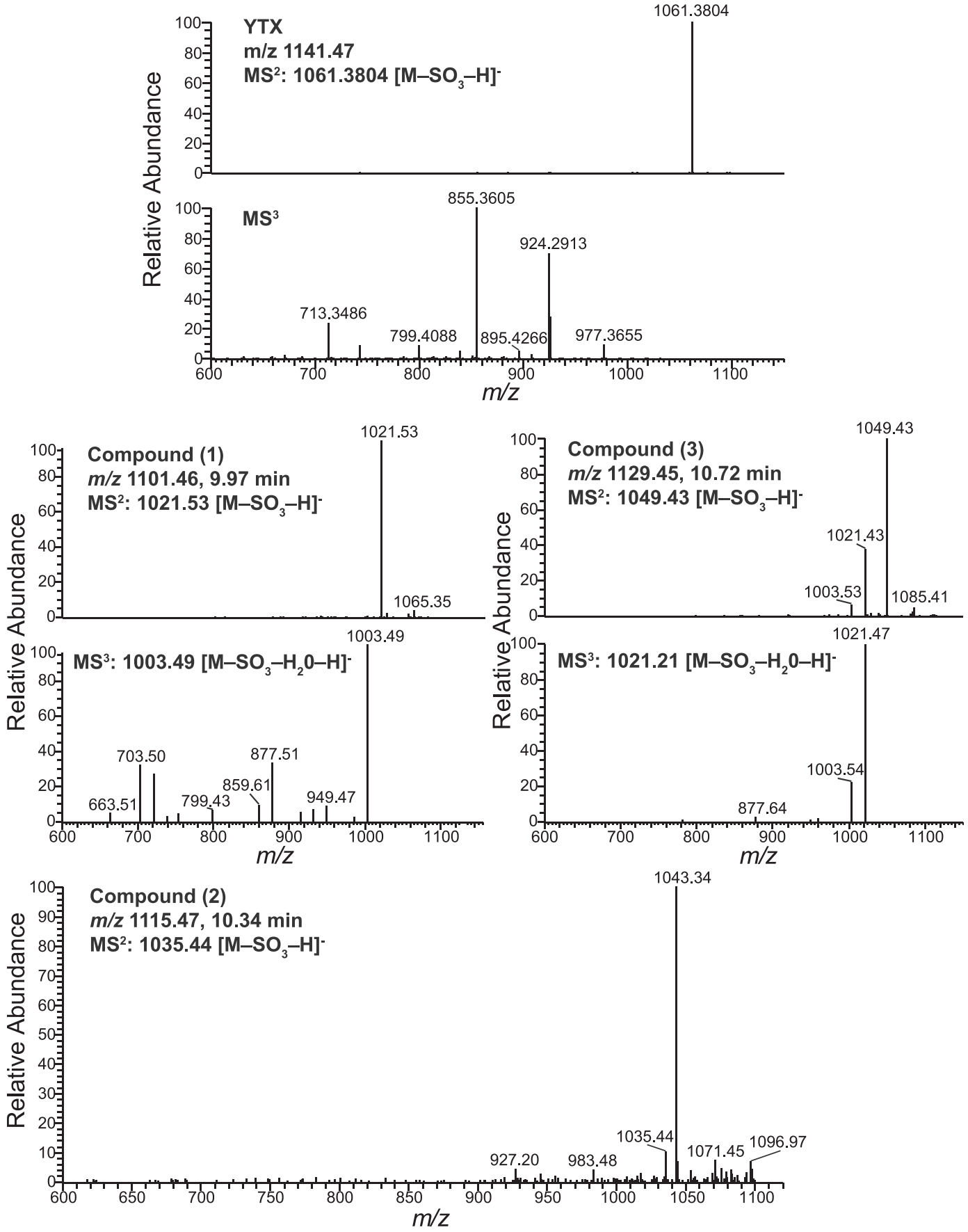

Fig. 7. Negative ion MS^2 and MS^3 fragmentation pattern for Compounds **1**–**3** from *Coolia malayensis* compared to yessotoxin (YTX).

fragmentation pattern for Compound **3** suggested that Compounds **1** and **3** might have an identical carbon skeleton. In contrast, the fragmentation pattern for Compound **2** was complex and the ion at *m/z* 1043.3 remains to be explained (Fig. 7). Interestingly, Compound **1** has an identical mass (1102.4) with 45,46,47-trinoryessotoxin (Satake et al., 1999); however, its MS^3 fragmentation pattern did not match with that of other yessotoxin analogs (Miles et al., 2006).

Supplementary Fig. S2 related to this article can be found, in the online version, at doi:10.1016/j.hal.2015.02.009.

In conclusion, compounds (**1**–**5**) identified in the dinoflagellate, *Coolia malayensis*, are composed of less oxygen compared to cooliatoxin and yessotoxin analogs, suggesting the metabolites produced by *C. malayensis* are unique to those previously reported. A micro-scale desalting (ZipTip C_{18}/P10) and HLB column (1 ml) desalting showed similar recovery of metabolites from water-soluble parts (Supplementary Fig. S3). These micro-scale desalting procedures have promise in primary screening of dinoflagellate metabolites and chemical profiling (a small scale culturing 1–5 L is enough for NanoLC–MS analysis). In addition, solid phase HLB and ODS extraction columns could be employed for large-scale desalting and fractionation work prior to HPLC purification. Large-scale cultivation, extraction, and purification work is necessary to fully characterize the chemistry and toxicity of these compounds.

Supplementary Fig. S3 related to this article can be found, in the online version, at doi:10.1016/j.hal.2015.02.009.

Authors' contribution

KCW isolated and cultured *Coolia malayensis*, and performed all the microscopy, DNA extraction and sequencing. AY performed the phylogenetic analyses. KCW and MCR performed chemical extractions on cultures. MCR performed all chemical analyses. KCW, AY and MCR interpreted the data. KCW, AY, MCR and HJ-K prepared the manuscript.

Acknowledgement

This study was completed with funding provided by the Okinawa Institute of Science and Technology, Okinawa, Japan.[SS]

References

Akasaka, K., Shichijyukari, S., Matsuoka, S., Murata, M., Meguro, H., Ohrui, H., 2000. Absolute configuration of a Ceramide with novel branched-chain fatty acid isolated from the epiphytic dinoflagellate, *Coolia monotis*. Biosci. Biotechnol. Biochem. 64, 1842–1846.

Aligizaki, K., Nikolaidis, G., 2006. The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. Harmful Algae 5, 717–730.

Armi, Z., Souad, T., Trabelsi, E., Maiz, N.B., 2010. First recorded proliferation of *Coolia monotis* (Meunier, 1919) in the North Lake of Tunis (Tunisia) correlation with environmental factors. Environ. Monit. Assess. 164, 423–433.

Dolapsakis, N.P., Kilpatrick, M.W., Economou-Amilli, A., Tafas, T., 2006. Morphology and rDNA phylogeny of a Mediterranean *Coolia monotis* (Dinophyceae) strain from Greece. Sci. Mar. 70, 67–76.

Faust, M.A., 1992. Observations on the morphology and sexual reproduction of *Coolia monotis* (Dinophyceae). J. Phycol. 28, 94–104.

Faust, M.A., 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. J. Phycol. 31, 996–1003.

Fraga, S., Penna, A., Bianconi, I., Paz, B., Zapata, M., 2008. *Coolia canariensis* sp. nov. (Dinophyceae), a new nontoxic epiphytic benthic dinoflagellate from the Canary Islands. J. Phycol. 44, 1060–1070.

Fukuyo, Y., 1981. Taxonomical study on benthic dinoflagellates collected in coral reefs. Bull. Jpn. Soc. Sci. Fish. 47, 967–978.

Holmes, M.J., Lewis, R.J., Jones, A., Wong Hoy, A.W., 1995. Cooliatoxin, the first toxin from *Coolia monotis* (Dinophyceae). Nat. Toxins 3, 355–362.

Howard, M.D.A., Smith, G.J., Kudela, R.M., 2009. Phylogenetic relationships of yessotoxin-producing dinoflagellates, based on the large subunit and internal trascribed spacer ribosomal DNA domains. Appl. Environ. Microbiol. 75, 54–63.

Ismael, A.A., 2014. First record of *Coolia monotis* Meunier along Alexandria coast – Egypt. Egypt. J. Aquat. Res. 40, 19–25.

Jeong, H.J., Yih, W., Kang, N.S., Lee, S.Y., Yoon, E.Y., Yoo, Y.D., Kim, H.S., Kim, J.H., 2012. First report of the epiphytic benthic dinoflagellates *Coolia canariensis* and *Coolia malayensis* in the waters off Jeju Island, Korea: morphology and rDNA sequences. J. Eukaryot. Microbiol. 59, 114–133.

Kogame, K., Horiguchi, T., Masuda, M., 1999. Phylogeny of the order Scytosiphonales (Phaeophyceae) based on DNA sequence of rbcL, partial rbcS, and partial LSU nrDNA. Phycologia 38, 496–502.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Laza-Martinez, A., Orive, E., Miguel, I., 2011. Morphological and genetic characterization of benthic dinoflagellates of the genera *Coolia*, Ostreopsis, and Prorocentrum from the south-eastern Bay of Biscay. Eur. J. Phycol. 46, 45–65.

Leaw, C.-P., Lim, P.-T., Chen, K.-W., Ng, B.-K., Usup, G., 2010. Morphology and molecular characterization of a new species of thecate benthic dinoflagellate, *Coolia malayensis* sp. nov. (Dinophyceae). J. Phycol. 46, 162–171.

Liang, J.-L., Long, L.-J., Zhang, S., Wu, J., 2009. Cooliatin, and unprecedented natural Dioxocyclononane from dinoflagellate *Coolia monotis* from South China Sea. Chem. Pharm. Bull. 57, 888–889.

Maddison, D.R., Wheeler, T.J., Maddison, W.P., 2007. Align: A Mesquite Package for Aligning Sequence Data. Version 1.11, http://mesquiteproject.org.

Meunier, A., 1919. Microplankton de la Mer Flamande. 111. Les Peridiniens. In: Hayez, M. (Ed.), Mem. Mus. R. Nat. Belgique Bruxelles 8 No. 1, p. 116.

Miles, C.O., Wilkins, A.L., Hawkes, A.D., Selwood, A.I., Jensen, D.J., Cooney, J.M., Buezenberg, V., MacKenzie, A.L., 2006. Identification of 45-hydroxy-46,47-dinoryessotoxin, 44-oxo-45,46,47-trinoryessotoxin, and 9-methyl-42,43,44,45,46,47,55-heptanor-38-en-41-oxoyessotoxin, and partial characterization of some minor yessotoxins, from *Protoceratium reticulatum*. Toxicon 47, 229–240.

Mohammad-Noor, N., Daugbjerg, N., Moestrup, Ø., Anton, A., 2004. Marine epibenthic dinoflagellates from Malaysia – a study of live cultures and preserved samples based on light and scanning electron microscopy. Nord. J. Bot. 24, 629–690.

Mohammad-Noor, N., Moestrup, Ø., Lundholm, N., Fraga, S., Adam, A., et al., 2013. Autecology and phylogeny of *Coolia tropicalis* and *Coolia malayensis* (dinophyceae), with emphasis on taxonomy of *C. tropicalis* based on light microscopy, scanning electron microscopy and LSU rDNA. J. Phycol. 49, 536–545.

Momigliano, P., Sparrow, L., Blair, D., Heimann, K., 2013. The diversity of *Coolia* spp. (Dinophyceae Ostreopsidaceae) in the Central Great Barrier Reef Region. PLOS ONE 8 (10), e79278.

Nakajima, I., Oshima, Y., Yasumoto, T., 1981. Toxicity of benthic dinoflagellates in Okinawa. Bull. Jpn. Soc. Sci. Fish. 47, 1029–1033.

Nakayama, T., Watanabe, S., Mitsui, K., Uchida, H., Inouye, I., 1996. The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. Phycol. Res. 44, 47–55.

Nunn, G.B., Theisen, B.F., Christensen, B., Arctander, P., 1996. Simplicity-correlated size growth of the nuclear 28S ribosomal RNA D3 expansion segment in the crustacean order Isopoda. J. Mol. Evol. 42, 211–223.

Pagliara, P., Caroppo, C., 2012. Toxicity assessment of *Amphidinium carterae*, Coolia cfr. monotis and Ostreopsis cfr. ovata (Dinophyta) isolated from the northern Ionian Sea (Mediterranean Sea). Toxicon 60, 1203–1214.

Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobó, P., Vernesi, C., 2005. Characterization of *Ostreopsis and Coolia* (Dinophyceae) isolates in the western Mediterranean sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. J. Phycol. 41, 212–225.

Rhodes, L.L., Thomas, A.E., 1997. *Coolia monotis* (Dinophyceae): a toxic epiphytic microalgal species found in New Zealand (Note). N. Z. J. Mar. Freshw. Res. 31, 139–141.

Rhodes, L.L., Smith, K.F., Munday, R., Selwood, A.I., McNabb, P.S., Holland, P.T., Bottein, M.-Y., 2010. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56, 751–758.

Rhodes, L., Smith, K., Papiol, G.G., Adamson, J., Harwood, T., Munday, R., 2014. Epiphytic dinoflagellates in sub-tropical New Zealand, in particular the genus *Coolia* Meuneir. Harmful Algae 34, 36–41.

Satake, M., Ichimura, T., Sekiguchi, K., Yoshimatsu, S., Oshima, Y., 1999. Confirmation of yessotoxin and 45,46,47-trinoryessotoxin production by *Protoceratium reticulatum* collected in Japan. Nat. Toxins 7, 147–150.

Satake, M., MacKenzie, L., Yasumoto, T., 1997. Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. Nat. Toxins 5, 164–167.

Shah, M.M.R., Samarakoon, K.W., Ko, J.-Y., Chaminda Lakmal, H.H., Lee, J.-H., An, S.-J., Jeon, Y.-J., Lee, J.-B., 2014. Potentiality of benthic dinoflagellate cultures and screening of their bioactivities in Jeju Island, Korea. Afr. J. Biotechnol. 13, 792–805.

Takano, Y., Horiguchi, T., 2006. Acquiring scanning electron microscopical, light miscroscopical and multiple gene sequence data from a single dinoflagellate cell. J. Phycol. 42, 251–256.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Tanaka, I., Matsuoka, S., Murata, M., Tachibana, K., 1998. A new Ceramide with a novel branched-chain fatty acid isolated from the epiphytic dinoflagellate *Coolia monotis*. J. Nat. Prod. 61, 685–688.

Ten-Hage, L., Turquet, J., Quod, J.P., Couté, A., 2000. *Coolia areolata* sp. nov. (Dinophyceae), a new sand-dwelling dinoflagellate from the southwestern Indian Ocean. Phycologia 39, 377–383.

Yasumoto, T., Seino, N., Murakami, Y., Murata, M., 1987. Toxins produced by benthic dinoflagellates. Biol. Bull. 172, 128–131.

Yasumoto, T., Yasukatsu, O., Murakami, Y., Nakajima, I., Bagnis, R., Fukuyo, Y., 1980. Toxicity of benthic dinoflagellates found in coral reef. Bull. Jpn. Soc. Sci. Fish. 46, 327–331.

Zingone, A., Siano, R., D'alelio, D., Sarno, D., 2006. Potentially toxic and harmful microalgae from coastal waters of the Campania region (Tyrrhenian Sea, Mediterranean Sea). Harmful Algae 5, 321–337.