

Diversity and toxicity of *Coolia* from the Ryukyu Islands, with a description of *Coolia kabiraensis* sp. nov

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ABSTRACT

Coolia is a widely distributed genus of marine benthic dinoflagellates, with some species known to produce bioactive compounds toxic to marine life. In this study, we investigated the diversity and toxicity of *Coolia* from the Ryukyu Islands (subtropical Japan), integrating morphological characterization, molecular phylogenetics, and *Artemia* toxicity assays. Five species were identified: four previously described, including the first record of *C. palmyrensis* in Japan, and one undescribed species, *Coolia kabiraensis* sp. nov. *Coolia kabiraensis* closely resembles members of the *Coolia monotis* species complex, which are difficult to distinguish morphologically. Phylogenetic analysis using ITS1–5.8S–ITS2 and 28S rDNA (D1–D3) genetic markers placed this taxon in a distinct, well-supported clade, supporting its recognition as a new species. A systematic review of global *Coolia* records, incorporating peer-reviewed studies, GenBank sequences, grey literature, and culture collections, was used to contextualize regional diversity. Species richness in the Ryukyu Islands was relatively high but comparable to that of other subtropical and tropical regions. Toxicity screening assays showed that extracts from *C. kabiraensis*, *C. palmyrensis*, *C. canariensis*, and *C. tropicalis* caused no statistically significant mortality of *Artemia* after 48 h. In contrast, *C. malayensis* extracts caused 40–85% mortality, indicating potential risk to marine life. These findings highlight the cryptic diversity, variable toxicity, and broad geographic distribution of *Coolia* species. Understanding species composition and toxicity patterns is essential for monitoring harmful algal blooms and anticipating future changes under climate-driven shifts in benthic microalgal assemblages.

1. Introduction

Marine benthic dinoflagellates inhabit diverse marine environments worldwide (Hoppenrath et al., 2023). Several genera, including *Coolia*, *Ostreopsis*, *Gambierdiscus*, *Amphidinium*, and *Prorocentrum*, can produce biotoxins that accumulate in seafood or impact marine organisms, contributing to fish kills, reduced fitness, and foodborne illness (Accoroni et al., 2011; Chinain et al., 2023; Mafra et al., 2023; Rolton et al., 2022; Sakamoto et al., 2021; Shears and Ross, 2009). These impacts may intensify with ocean warming and shifting oceanographic conditions (Gobler, 2020; Tester et al., 2020).

Coolia has been reported from tropical, subtropical and temperate coastal waters, but many island regions are scarcely documented. Identification is complicated by morphological similarity between

species, limiting understanding of diversity and distribution (Hoppenrath et al., 2023; Leaw et al., 2010, 2016). Phylogenetic analyses, particularly using the large subunit (28S) ribosomal RNA genes and the internal transcribed spacer region (ITS), have improved species resolution for *Coolia* (Leaw et al., 2016; Phua et al., 2021; Rhodes et al., 2014; Wakeman et al., 2015). *Coolia* commonly co-occurs with other toxic benthic dinoflagellates (Aligizaki and Nikolaidis, 2006; Morton et al., 1992) making attribution of toxic events difficult. While *Gambierdiscus* is more strongly associated with seafood poisoning (Chinain et al., 2023; Parsons et al., 2012; Perkins et al., 2024; Stuart et al., 2022), mixed assemblages may pose cumulative risks.

Toxicity in *Coolia* is highly variable across species, phylogroups, and even strains, complicating ecological and public health assessments (Leung et al., 2017; Miralha et al., 2023). While some strains produce

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toxins structurally similar to those of other dinoflagellates (e.g., cooliatoxin and gambierone), others of the same species show no detectable toxicity (Holmes et al., 1995; Leaw et al., 2016; Rhodes et al., 2014; Tibirić et al., 2020). Laboratory extracts nonetheless show toxic effects across different test organisms (Karafas et al., 2015; Rhodes and Thomas, 1997). Compared to *Gambierdiscus* and *Ostreopsis*, whose health impacts are well recognised, the ecological and toxicological significance of *Coolia* requires further investigation.

The Ryukyu Islands of southern Japan have a subtropical climate and support diverse marine ecosystems. The region has a history of ciguatera fish poisoning, with *Gambierdiscus* recognised as the primary cause (Oshiro et al., 2010), and is consequently well studied in Japan (Funaki et al., 2022; Kuno et al., 2010; Nishimura et al., 2016, 2013; Yoshimatsu et al., 2014). In contrast, toxin-producing benthic dinoflagellates such as *Coolia* remain understudied (Faust, 1995; Wakeman et al., 2015). Identifying these taxa and their toxicity is essential for evaluating risks to public health and marine ecosystems.

To this end, the present study investigated the diversity of *Coolia* in the Ryukyu Islands, compared these findings with global diversity patterns, and examined the potential toxic effects of local strains. Five species, including a new genetically distinct taxon, were examined using morphological and molecular approaches. Records from past literature, GenBank, and culture collections were also compiled to contextualise species diversity results, and the toxicity of methanolic extracts was assessed using *Artemia* bioassays to evaluate potential ecological and public health risks.

2. Methods

2.1. Study area and sample collection

Sediment samples were collected from low intertidal and shallow subtidal zones by taking the upper 5 cm of sandy sediment into 500 mL plastic containers from two sites in Okinawa Prefecture, southern Japan, between January 2021 and September 2023. Site 1 was Odo Beach in southern Okinawa (26.08872°N, 127.70885°E) and Site 2 was Kabira Bay on Ishigaki Island (24.46908°N, 124.14217°E). Collected sediment samples were sealed in airtight plastic containers, kept cold, and transported to the laboratory within 6 h for further processing.

2.2. Culture establishment

Upon arrival at the laboratory, sediment samples were processed to establish crude cultures. Approximately 5 g of collected surface sediment was added to sterile plastic containers (200 mL capacity) containing 50 mL of filtered and autoclaved seawater at 32 salinity, supplemented with half-strength Daigo's IMK medium (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan).

The crude cultures were incubated at 25°C with a 12:12 light-dark cycle. After 24 h, single *Coolia* cells were isolated from the crude cultures using an inverted microscope (Olympus CKX53, Olympus, Tokyo, Japan) and the hand-drawn capillary pipette method described by Andersen and Kawachi (2005). Individual cells were transferred into wells of 24-well plates containing 0.22 µm-filtered and autoclaved seawater supplemented with half-strength Daigo's IMK growth media. Isolated cultures were incubated at 25°C, with 80 µmol photons m⁻² s⁻¹ at a 12:12 light-dark cycle, and once visible cell density was reached and cultures were confirmed to be free from contaminants, they were transferred to 30 mL petri dishes containing fresh half-strength IMK growth media. Cultures were maintained by sub-culturing into fresh media every 30 days.

2.3. Morphological analysis

Coolia species isolated in this study were analyzed using light and scanning electron microscopy (SEM). For light microscopy, live cells

were mounted on glass slides and imaged using a Zeiss Axioskop 2 Plus microscope (Zeiss, Oberkochen, Germany) connected to a Canon EOS Kiss X8i digital camera (Canon, Tokyo, Japan). For SEM, motile stages of *Coolia* strain C14.2 were fixed with 1 % glutaraldehyde, filtered onto 2 µm pore size MF-millipore membrane filters (Merck, Darmstadt, Germany), washed twice with distilled water, and dehydrated through a graded ethanol series (30%, 40%, 50%, 60%, 70%, 80%, 90%, and three changes of 100%) for 10 min at each step. Samples were then critical-point dried (Leica EM CPD300, Leica, Germany), mounted on SEM stubs, sputter-coated with 5 nm gold, and imaged using a Hitachi N-3000 scanning electron microscope. For confocal laser scanning microscopy, *Coolia* strain C14.2 cells were stained with 0.025 mg/mL of Calcofluor White (Sigma-Aldrich, St. Louis, MO, USA), mounted on glass slides, and imaged with a Zeiss LSM 980 laser scanning microscope (Zeiss, Oberkochen, Germany). Additionally, cells were gently squashed under coverslips, dried overnight, then stained with 0.025 mg/mL of Calcofluor White and examined using epifluorescence microscopy to improve visualisation of the cingular and sulcal regions. This approach enhanced the visibility of cingular plate boundaries but did not fully resolve individual sulcal plates.

2.4. DNA extraction, amplification and sequencing

Genomic DNA was extracted from cultures using the Masterpure™ Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI, USA) following the manufacturer's protocol. The internal transcribed spacer region (ITS1–5.8S–ITS2), and the D1–D3 region of the large subunit (28S) rDNA were targeted for amplification.

Initial amplification of ITS and 28S rDNA was performed using KOD One™ Blue polymerase (Toyobo, Osaka, Japan) with the primer pair SR1/28-1483R or with D1RF1/28-1483R (Table S1). Thermocycler conditions for the initial amplification were as follows: initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 5 s, and extension at 68°C for 20 s; followed by a final extension at 68°C for 5 min. A nested PCR reaction followed using 1:50 times diluted PCR products as the template. The primer pair Lp1F1/25F1R was used to amplify the ITS region and the primer pair D1RF1/852R-70 was used for the 28S rDNA region (Table S1). Thermocycler conditions were: initial denaturation at 94°C for 1 min; 30 cycles of denaturation at 98°C for 10 s, 54/55°C (ITS/28S) for 5 s, and 68°C for 10 s; followed by a final extension at 68°C for 5 min.

The PCR products were checked for size and quality on a 1% agarose gel stained with ethidium bromide. They were then purified using polyethylene glycol (PEG) precipitation, dye-labeled with Brilliant Dye™ Terminator v1.1 (NimaGen, Nijmegen, Netherlands) following the manufacturer's protocol and sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Massachusetts, USA). Sequences generated in this study were deposited in NCBI GenBank (accession numbers listed in Table S2).

2.5. Phylogenetic analysis

Sequencing reads were trimmed, assembled with the 'De Novo Assemble' tool, and manually checked using Geneious Prime v2025.1.2 (Kearse et al., 2012). Preliminary species identification was performed using the NCBI Basic Local Alignment Search Tool (BLAST), comparing sequences from this study against available *Coolia* sequences in GenBank. Similar sequences and additional reference sequences retrieved from GenBank were compiled and sorted into two datasets: ITS (ITS1–5.8S–ITS2 region) and 28S rDNA (D1–D3).

Final alignment files were made using the Multiple Alignment using Fast Fourier Transform (MAFFT v7.490; Katoh and Standley, 2013) implemented within Geneious Prime. Over hanging base pair ends were manually trimmed, bringing the final length to 427 bp and 778 bp for the ITS and 28S rDNA respectively.

The program IQ-TREE v2.4.0 (Minh et al., 2020) was used to

determine the best-fit nucleotide substitution models based on the Akaike Information Criterion corrected (AICc) and the Bayesian Information Criterion (BIC). Phylogenetic trees were constructed using Maximum Likelihood (ML) and Bayesian inference methods. ML analyses were conducted with 1000 bootstrap replicates following standard analysis (Felsenstein, 1985). Bayesian analyses were performed in MrBayes v3.2.7a (Ronquist et al., 2012), sampling every 100 generations from four Markov Chain Monte Carlo (MCMC) chains over a total of 10 million generations. Convergence of MCMC chains was assessed using the coda package v0.19–4.1 (Plummer et al., 2006) in R v4.4.2 (R Core Team, 2024).

Maximum Likelihood and Bayesian consensus trees were visualized and annotated using the ggtree package v3.14.0 (Yu et al., 2018) in R v4.4.2. Branch supports were mapped onto the respective tree topologies (bootstrap values for ML; posterior probabilities for Bayesian inference). Pairwise genetic distances among sequences were calculated under the Kimura 2-parameter (K80) model using the ape package v5.8-1 (Paradis et al., 2004) in R to characterize intraspecific and interspecific divergence. Additionally, ITS2 secondary structures of *Coolia* strain C14.2 and strains of closely related species were predicted using RNAfold ViennaRNA package (Lorenz et al., 2011). Minimum free energy (MFE) structures were inspected for comparative analysis. Compensatory base changes were identified and compared manually. ITS2 boundaries were defined based on the annotated ITS2 region of *C. malayensis* (AF244943) following sequence alignment of ITS sequences.

2.6. Biogeographic data compilation and mapping

Distribution data for *Coolia* species were compiled from peer-reviewed publications, GenBank sequence records, grey literature, and public culture collections. Only records supported by molecular data (ITS and 28S rDNA sequences) and/or clear morphological identification were included. Exceptions were made for under-sampled regions (e.g., North-eastern Africa) as including these records was important for assessing *Coolia*'s distribution. Species names were updated where necessary to reflect recent phylogenetic revisions. Compiled records were mapped by geographic locality to visualize global species distributions. The database comprises 517 unique records, including those gathered in the present study for the Ryukyu Islands (Table S3). Species occurrence mapping was conducted in R v4.4.2 using the ggplot2 (Wickham, 2016), sf (Pebesma, 2018; Pebesma and Bivand, 2023), and rnaturalearth (Massicotte and South, 2025) packages, plotting species occurrences overlaid on Natural Earth base maps.

2.7. Toxin extraction and sample preparation

For toxin extraction, *Coolia* strains were cultured as above but in larger glass petri dishes containing 50 mL full-strength Diago's IMK medium. A total of 10 petri dishes per strain were seeded from their corresponding stock culture (500 mL total volume bulk culture per strain). Bulk cultures were incubated until reaching stationary phase (~21 days; cell density $\sim 2.1 \times 10^4$ cells per mL) and were observed periodically to assess cell health and detect contamination. Cultures were vacuum filtered onto Whatman 1823-047 GF/D glass microfiber filters (~0.89 g of wet weight per algal pellet). Filters were transferred to separate 50 mL Falcon tubes and stored at -80°C until extraction.

Filters were thawed overnight at 4°C prior to methanol extraction. Each filter was submerged in 10 mL methanol by brief centrifugation and soaked overnight at 4°C . The methanol extract was then transferred into a new Falcon tube. An additional 10 mL of methanol was added to each filter, stirred, soaked for 2 h, and centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was collected and combined with the initial extract. A final overnight methanol soak and centrifugation was performed, and the resulting supernatant pooled. Methanol was evaporated under reduced pressure using a rotary evaporator (AS ONE, Osaka,

Japan) and the crude extracts were stored at -80°C .

Desalting was performed using Supel™-Select HLB 1 mL tubes (Merck, Darmstadt, Germany). Columns were conditioned with 1 mL methanol, and samples were dissolved in aqueous methanol (90:10 water:methanol, v/v) before loading onto the columns. Columns were washed with 1 mL aqueous methanol (90:10, v/v), and retained compounds were eluted with 1–2 mL pure methanol. The final methanol extracts were dried under reduced pressure with the rotary evaporator and stored at -80°C for later use in bioassays.

2.8. Toxicity screening bioassay

To screen for toxicity, brine shrimp (*Artemia* spp., Spectrum Brands Japan Co., Yokohama, Japan) eggs were hatched in 0.22 μm -filtered and autoclaved seawater at 25°C . After 24 h of incubation, 10 hatched nauplii were transferred into individual wells of 24-well plates containing 475 μL of filtered seawater. Crude extracts were dissolved in 100 μL of 1% (v/v) dimethyl sulfoxide (DMSO) and 25 μL of extract solution were added to each well to achieve a final volume of 500 μL . Four replicates of 10 shrimp were prepared per strain treatment ($n = 40$).

Live *Artemia* were counted at 12, 24, and 48 h intervals. Behavioral observations (e.g., reduced swimming activity, erratic movements) were also recorded qualitatively. Two control treatments were included: (1) seawater control (25 μL filtered seawater added), and (2) solvent control (25 μL 1% DMSO in seawater added). As mortality was negligible and behavior normal in both control groups, data from the solvent control were used for all statistical analyses and presentation of control mortality. No food was provided during the assay; however, control mortality remained negligible throughout the 48 h observation period.

2.9. Statistical analysis

Mortality proportion data were arcsine square root-transformed prior to analysis to meet assumptions of parametric tests. One-way ANOVA for independent samples was applied to compare survival proportions of *Artemia* nauplii among the control and treatment groups after 24 and 48 h of incubation. Shapiro–Wilk and Levene's tests were used to assess the normality of residuals and homogeneity of variances, respectively. Dunnett's post hoc test was applied to compare each treatment group to the control. Results were considered statistically significant at $p \leq 0.05$. All statistical analyses were conducted using R v4.4.2 (R Core Team, 2024). Data processing and visualization were performed using tidyverse v2.0.0 (Wickham et al., 2019) and ggplot2 (Wickham, 2016) packages, respectively. Assumption testing and post hoc analyses were conducted using the car v3.1–3 (Fox and Weisberg, 2019) and multcomp v1.4–28 (Hothorn et al., 2008) packages.

3. Results

3.1. Morphology of *Coolia* species

The general morphology of *C. malayensis*, *C. palmyrensis*, *C. canariensis*, and *C. tropicalis* strains isolated in this study was consistent with previously published descriptions. Light micrographs of representative strains are shown in Fig. 1. Detailed morphological observations of the novel species (*Coolia kabiraensis*) are presented below.

***Coolia kabiraensis* S. Cunningham, S. Riewluang, A. Yamaguchi, A. Rolton & K.C. Wakeman sp. nov.** (Figs. 1A and B, 2, and 3)

Etymology: the epithet refers to Kabira Bay, Ishigaki Island, the type locality of the species.

Type locality: Kabira Bay, Ishigaki Island, Okinawa Prefecture, Japan (24.46908°N, 124.14217°E).

Distribution: Subtropical region of Okinawa Prefecture in Japan, southwest of Okinawa Island.

Ecology: *Coolia kabiraensis* was isolated from subtidal sandy substrate in Kabira Bay located on the north coast of Ishigaki Island,

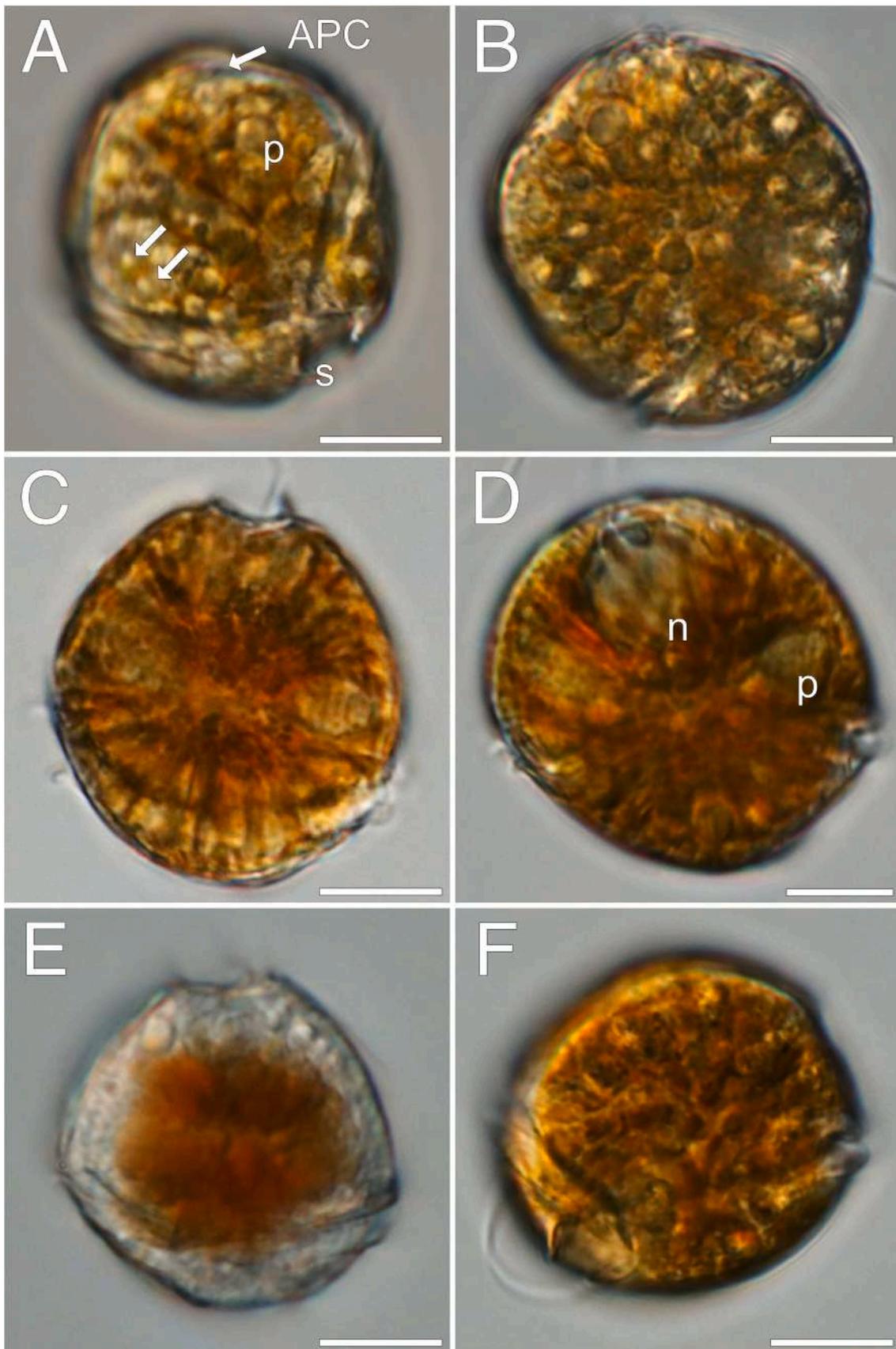


Fig. 1. Light micrographs of *Coolia* spp. showing general cell morphology. (A-B) *Coolia kabiraensis*: globular cell with visible chloroplasts; (A) shows the sulcus, thecal pores, and apical pore complex. (C) *C. canariensis*. (D) *C. tropicalis* with visible dorsal nucleus and pusule. (E) *C. palmyrensis*. (F) *C. malayensis*. Abbreviations: s, sulcus; p, pusule; n, nucleus; APC, apical pore complex. Scale bars = 10 μ m.

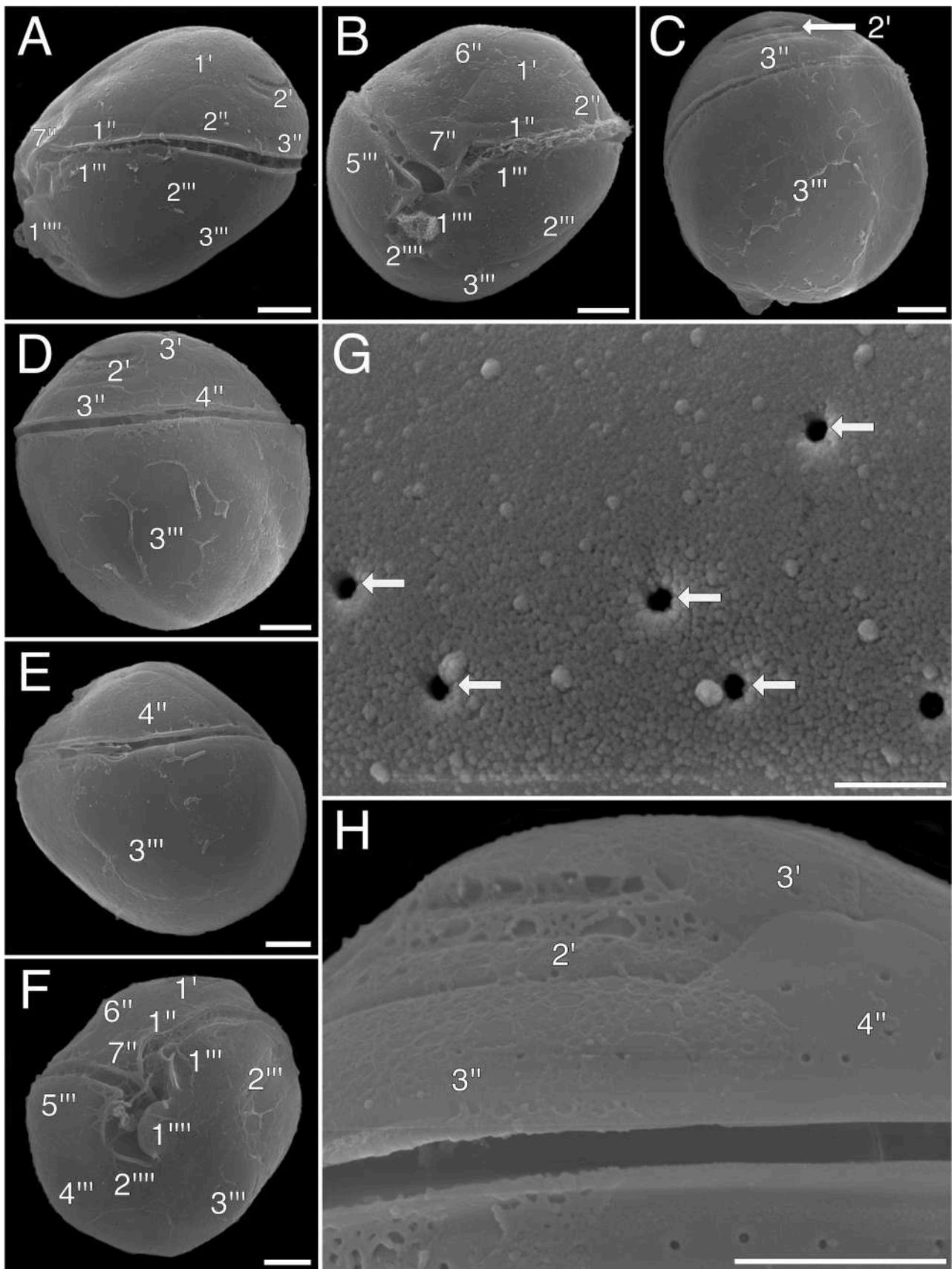


Fig. 2. Scanning electron micrographs of *Coolia kabiraensis* showing thecal tabulation. (A) Left lateral view. (B) Ventral view. (C) Dorsal view of hypotheca with 3'' and 2' plates of epitheca (bearing the apical pore complex) visible. (D) Dorsal to right lateral view. (E) Right lateral view. (F) Ventral view. (G) Detail of thecal pores (arrows). (H) Detail of the dorsal epitheca. All scale bars = 5 μ m except (G) = 1 μ m.

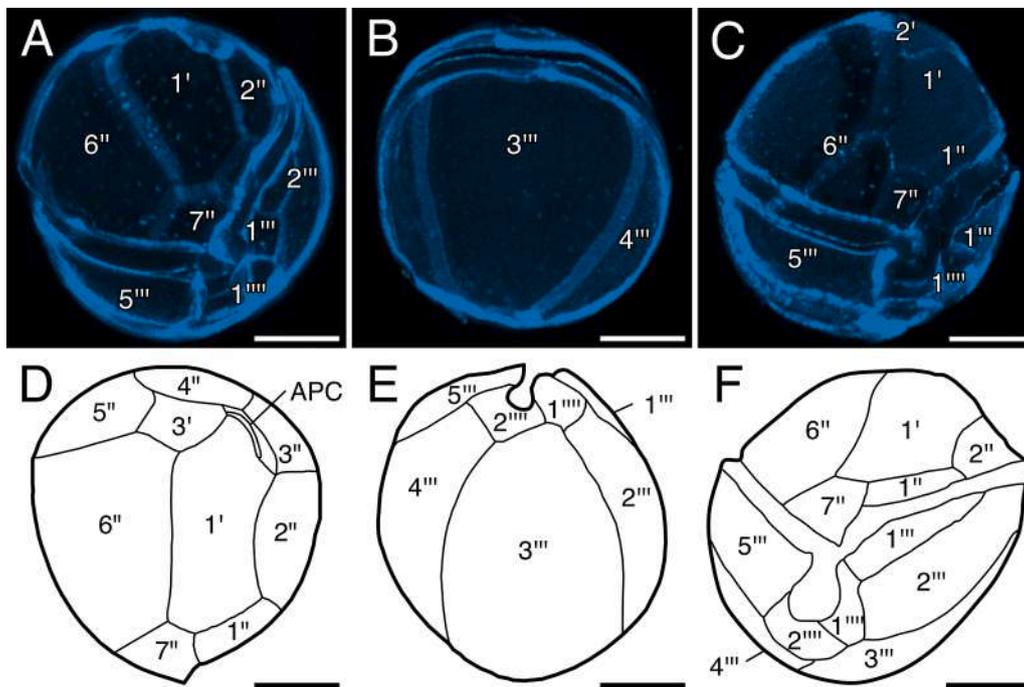


Fig. 3. Confocal laser scanning images and line drawings of *Coolia kabiraensis* showing thecal plate patterns. (A) Apical-ventral view. (B) Hypotheca-dorsal view. (C) Ventral view. Line drawing: (D) Apical view. (E) Hypotheca view. (F) Ventral view. Line drawings adapted from SEM and confocal images. Scale bars = 10 μm .

Okinawa Prefecture, Japan. The bay forms part of the Iriomote-Ishigaki National Park. *Coolia kabiraensis* was found associated with other *Coolia* species and in the digestive syncytium (gut tissue) of a symbiotic acoele.

Gene sequence: The ITS (ITS1–5.8S–ITS2) and partial 28S rDNA sequences of *Coolia* strain C14.2 has been submitted to the GenBank database under the accession numbers: OQ453219 (ITS), OQ453218 (28S).

Holotype: Fixed and dried specimens on SEM stub (specimen code: C14.2), held in the Biodiversity Laboratory associated with the Hokkaido University Museum, Sapporo, Japan.

Reference material: Living cultures are maintained in the Biodiversity Laboratory associated with the Hokkaido University Museum, Sapporo, Japan (culture name: C14.2).

Diagnosis: Cell shape is round, 26–42 μm in length and 27–39 μm in width (Fig. 1A and B). The plate formula is Po, 3', 7'', 6–7c?, ?s, 5''', 2'''. The apical pore is 5–8 μm in length and inside a relatively elongate second apical (2') plate. Thecal pores are small and uniform in size.

Morphologically similar to species of the *C. monotis* complex (*C. monotis*, *C. malayensis*, *C. santacroce*, and *C. palmyrensis*). Distinguished from all other genetically described *Coolia* species based on species-level sequence divergence of the ITS and partial 28S rDNA (D1–D3).

Description: The species *C. kabiraensis* sp. nov., was characterized based on cells obtained from cultures (strain C14.2). Cells measured 26–42 μm in length (mean = 31.5 \pm 3.5 μm , n = 30), 27–40 μm in width (mean = 30.9 \pm 3.1 μm , n = 28), and 22–37 μm in dorsoventral depth (mean = 30.1 \pm 3.5 μm , n = 16). Cells are nearly round in shape with numerous golden-brown peridinin-containing chloroplasts distributed throughout the cell (Fig. 1A and B). The epitheca was slightly smaller than the hypotheca (Fig. 2). Plate 1' is narrow and oblong. Plate 6'' is the largest epithecal plate, and 7'' is small and five-sided. The apical pore complex is slightly curved and measures 5–7 μm in length (mean = 5.9 \pm 0.8 μm , n = 10). It is located on the 2' plate, which measures 8–11 μm in length (mean = 9.6 \pm 0.8 μm , n = 8). The apical pore is surrounded by the 1', 2' and 3' plates (Fig. 2A, D, H). The 3' plate is five-sided. Cingular plate boundaries were partially resolved under SEM and epifluorescence microscopy, allowing 6–7 cingulum plates to be estimated, but

individual sulcal plates could not be clearly distinguished (Figure S1 and S2). Plate 3''' is the largest plate of the hypotheca (Fig. 2C–E). Plate 2''' is in contact with 1''' but not 2'' (Fig. 2B and F). The thecal surface of the epitheca and hypotheca is smooth and covered with scattered round thecal pores 0.16–0.30 μm (mean = 0.23 \pm 0.03 μm , n = 90). Narrow descending cingulum, and the sulcus was bordered by sulcal lists extending from plates 1'', 5'', 1''' and 2'''. Morphological description is supported by light (Fig. 1A and B), scanning electron (Fig. 2) and confocal (Fig. 3A–C) micrographs, and is also summarized by a line drawing of the plate tabulation (Fig. 3D–E).

Remarks: *Coolia kabiraensis* is distinguished from other members in the *Coolia monotis* complex primarily by molecular evidence, with no conspicuous morphological characters that separate it from closely related taxa (Fig. 1 and Table S4). *Coolia kabiraensis* is morphologically consistent with other species in the *C. monotis* complex, particularly *C. malayensis*, *C. palmyrensis*, and *C. santacroce*. On average, *C. palmyrensis* has a smaller cell size than all other *C. monotis* complex species (Fig. 1 and Table S4). All species share a round to lens-shaped cell outline, smooth thecal surface, narrow descending cingulum, and similar plate arrangements: plate 6'' is the largest in the epitheca, 3''' is the largest in the hypotheca, and plate 2''' contacts 1''' but not 2''. Measured traits such as cell size, apical pore length, and thecal pore diameter overlap across species, although *C. kabiraensis* had slightly larger mean cell dimensions than *C. palmyrensis* and *C. santacroce*, and closely matched *C. malayensis*. The 7'' plate is small and five-sided, the 2' plate is $\sim 1.5 \times$ the apical pore length, and pores are scattered, round to oval, with no distinct ornamentation. Due to the high degree of morphological similarity, molecular data are essential to reliably distinguish *Coolia kabiraensis* from its closest relatives within the *C. monotis* complex.

3.2. Phylogenetics

Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic analyses performed on the ITS region and 28S rDNA resolved eight distinct species-level clades across both markers. *Coolia kabiraensis* was consistently resolved as a distinct lineage within the *C. monotis* complex.

In the ITS tree it branched closest to *C. palmyrensis* with moderate support (ML = 64 BI = 0.99, Fig. 4). In the 28S rDNA tree, it diverged from the remainder of the complex (*C. monotis*, *C. malayensis* and *C. santacroce*) with strong support (ML = 95, BI = 1, Fig. 5). This new lineage clustered within the *C. monotis* species complex and exhibited 17–32% (ITS) and 13–19% (28S) uncorrected p-distance from other members of the complex (Table S5 and S6). For the 28S rDNA analysis it was closest to *C. santacroce* at 13%. *C. kabiraensis* predicted ITS2 secondary structures differed from closely related species (Figure S3). Manual comparison of paired nucleotide sites showed several compensatory base changes between *C. kabiraensis* and *C. malayensis* (AF244943), whereas *C. malayensis* (strain Cm18 from this study) exhibited zero compensatory base changes in comparison to AF244943.

Reference sequences of *C. guanchica*, *C. monotis* and *C. santacroce* were included for comparison, although no strains of these species were isolated in the present study. *C. areolata*, which lacks published sequence data, was not included in molecular analyses as its description is based solely on morphology.

All strains from this study clustered with support within their respective species clades in both markers (Figs. 4 and 5), confirming species identities and expanding the genetic data set for *Coolia* in Japan.

3.3. Biogeography

The global distribution of known *Coolia* species, including the results of the present study, is shown in Fig. 6 and Table 1. *C. malayensis* was the most frequently reported and widely distributed species, whereas reports of *C. monotis* were restricted to the North Atlantic and Mediterranean. *C. tropicalis* occurred predominantly at lower latitudes but was reported across multiple ocean basins. Both *C. guanchica* and *C. kabiraensis* were each reported from a single geographic area. Compiled records illustrate that *Coolia* species are predominantly distributed across tropical and subtropical regions, with some taxa (i.e., *C. monotis* and *C. malayensis*) extending into higher latitudes.

3.4. Toxicity of methanolic extracts of *Coolia* spp. to brine shrimp

A one-way ANOVA revealed a significant effect of strain on *Artemia* mortality at 48 h (ANOVA, $F_{20,63} = 28.97$, $p < 0.001$). After 48 h exposure, of the 20 methanolic extracts tested, all nine strains of *C. malayensis*, caused significant mortality of brine shrimp compared to controls ($p < 0.001$, Table S7, Fig. 7). *Artemia* mortality rates in these *C. malayensis* treatments ranged from ~42% to 83% after 48 h, there were statistically non-significant mortalities in other *Coolia* treatments (*C. canariensis*, *C. palmyrensis*, *C. tropicalis* and *C. kabiraensis* did not exceed 15% *Artemia* mortality), and there was no mortality in the controls.

In addition to mortality, behavioral changes were observed in treatment groups. Between 12–24 h post-exposure, nauplii exposed to *C. malayensis* extracts displayed abnormal movements such as jittery motion, sluggishness, and clustering. By 48 h, some individuals exposed to extracts of *C. canariensis* strain Cc13, *C. palmyrensis* strain Cp64 and *C. tropicalis* strain Ct6 exhibited signs of impaired swimming (e.g., imbalance or reduced mobility), though without substantial mortality.

4. Discussion

We identified five distinct *Coolia* species from the Ryukyu Islands, including the first confirmed record of *C. palmyrensis* in Japan, and report a novel species, *C. kabiraensis*. Isolates of *C. canariensis* and *C. tropicalis* were morphologically distinct from species within the *Coolia monotis* complex (*C. malayensis*, *C. palmyrensis* and *C. kabiraensis*). Within the *monotis* complex however, morphological differences were subtle and often not diagnostic, reinforcing the need for genetic analysis. Phylogenetic results placed *C. kabiraensis* in a distinct clade within the complex. *Coolia* diversity in the Ryukyu Islands was high, but

comparable to other tropical and subtropical regions. Toxicity screening assays showed pronounced effects from *C. malayensis*, while other species caused minimal mortality.

4.1. *Coolia* taxonomy, species determination and diversity

Coolia species are grouped into three phylogenetic clades or morphological groups: the *C. monotis* complex, the *C. canariensis* complex, and *C. tropicalis* (David et al., 2014). Within the *C. monotis* complex, morphological differences are subtle, variable, or absent, making species delineation based on morphology alone unreliable (David et al., 2020; Hoppenrath et al., 2023; Karafas and Tomas, 2015; Leaw et al., 2016). While some studies have proposed using traits like cell size, apical pore length, or thecal pore structure to differentiate species (Karafas et al., 2015; Leaw et al., 2010; Momigliano et al., 2013), these features often vary within species or are shared across taxa, and molecular evidence is required. For example, a matrix structure in thecal pores was initially diagnostic for *C. malayensis* (Leaw et al., 2010), but has since been observed in *C. monotis* (David et al., 2014), *C. canariensis* (Jeong et al., 2012), *C. palmyrensis* (Karafas et al., 2015), and even in other genera such as *Fukuyoa* (Laza-Martínez et al., 2016) and *Ostreopsis* (David et al., 2013).

Among gonyaulacoid taxa (of which *Coolia* is a genus) different thecal plate interpretations exist, and different tabulation systems have been used in the literature. For *Coolia*, most taxonomic treatments, including species descriptions of *C. tropicalis* (Faust, 1995), *C. areolata* (Ten-Hage et al., 2000), *C. canariensis* (Fraga et al., 2008), *C. malayensis* (Leaw et al., 2010), and *C. palmyrensis* and *C. santacroce* (Karafas et al., 2015) follow the modified Kofoidian interpretation (Fensome et al., 1993; Hoppenrath et al., 2023). Some authors have applied the homology-based interpretation of Po, 4', 6'' epitheca tabulation from Besada et al. (1982) for gonyaulacoid taxa (e.g., David et al., 2020; Fraga et al., 2011) which is useful for comparison across genera. For consistency with the majority of *Coolia* species descriptions and because our specimens show discrete apical plates surrounding the apical pore which do not contact the cingulum, we applied the Po, 3', 7'' tabulation of the epitheca.

Sulcal plates of *C. kabiraensis* could not be easily distinguished in the present study. Recent work on gonyaulacoid taxa highlights the value of ventral-area characters for resolving plate homologies and taxonomic boundaries (Carbonell-Moore et al., 2022). To this end, cingular and especially sulcal plates have been reported inconsistently across *Coolia* species descriptions. For example, the sulcal plates in *C. areolata* (Ten-Hage et al., 2000), *C. guanchica* (David et al., 2020), *C. malayensis* (Leaw et al., 2010), *C. monotis* (David et al., 2014), *C. palmyrensis* (Karafas et al., 2015), *C. santacroce* (Karafas et al., 2015) and *C. tropicalis* (Faust, 1995) are not well defined, making direct and detailed taxonomic comparisons using this particular morphological character challenging.

Coolia kabiraensis formed a distinct clade. The 28S rDNA dataset placed *C. kabiraensis* sister to a clade containing *C. monotis*, *C. malayensis*, and *C. santacroce*. The ITS region resulted in a topology where *C. kabiraensis* is sister to *C. palmyrensis*, albeit with lower statistical support. Both phylogenetic analyses produced topologies consistent with previous studies (David et al., 2020; Karafas et al., 2015; Phua et al., 2021; Verma et al., 2023; Wakeman et al., 2015), and support the placement of *C. monotis*, *C. malayensis*, *C. palmyrensis*, *C. santacroce*, and *C. kabiraensis* within a closely related group. Genetic distances observed among *Coolia* species in this study are consistent with previous reports (David et al., 2020; Karafas et al., 2015; Leaw et al., 2016; Momigliano et al., 2013). Species within the *C. monotis* complex often differ only slightly in 28S rDNA sequences but exhibit multiple compensatory base changes in ITS2 secondary structure, a recognized indicator of species-level divergence (Leaw et al., 2016). These species also occupy different environmental niches and vary in toxicity, as supported by the present study.

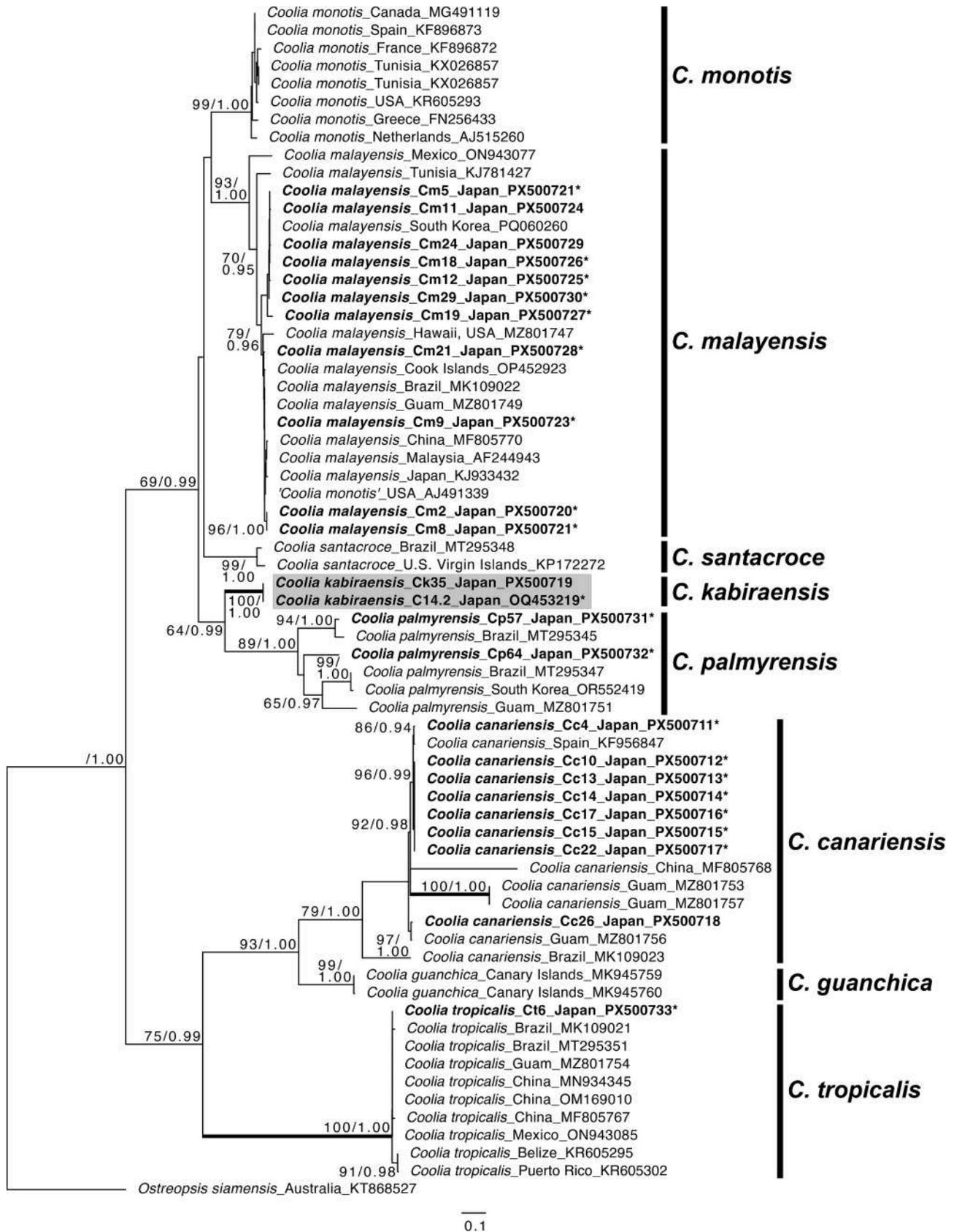


Fig. 4. Maximum likelihood (ML) phylogenetic tree of *Coolia* strains based on ITS (ITS/5.8S) rDNA sequences. Numbers at nodes indicate ML bootstrap support (1000 pseudo replicates) and Bayesian Inference (BI) posterior probabilities. Strains isolated in this study are shown in bold. Sequences of *Coolia kabiraensis* are highlighted in bold with a grey background. Asterisks (*) indicate strains used for *Artemia* toxicity bioassay.

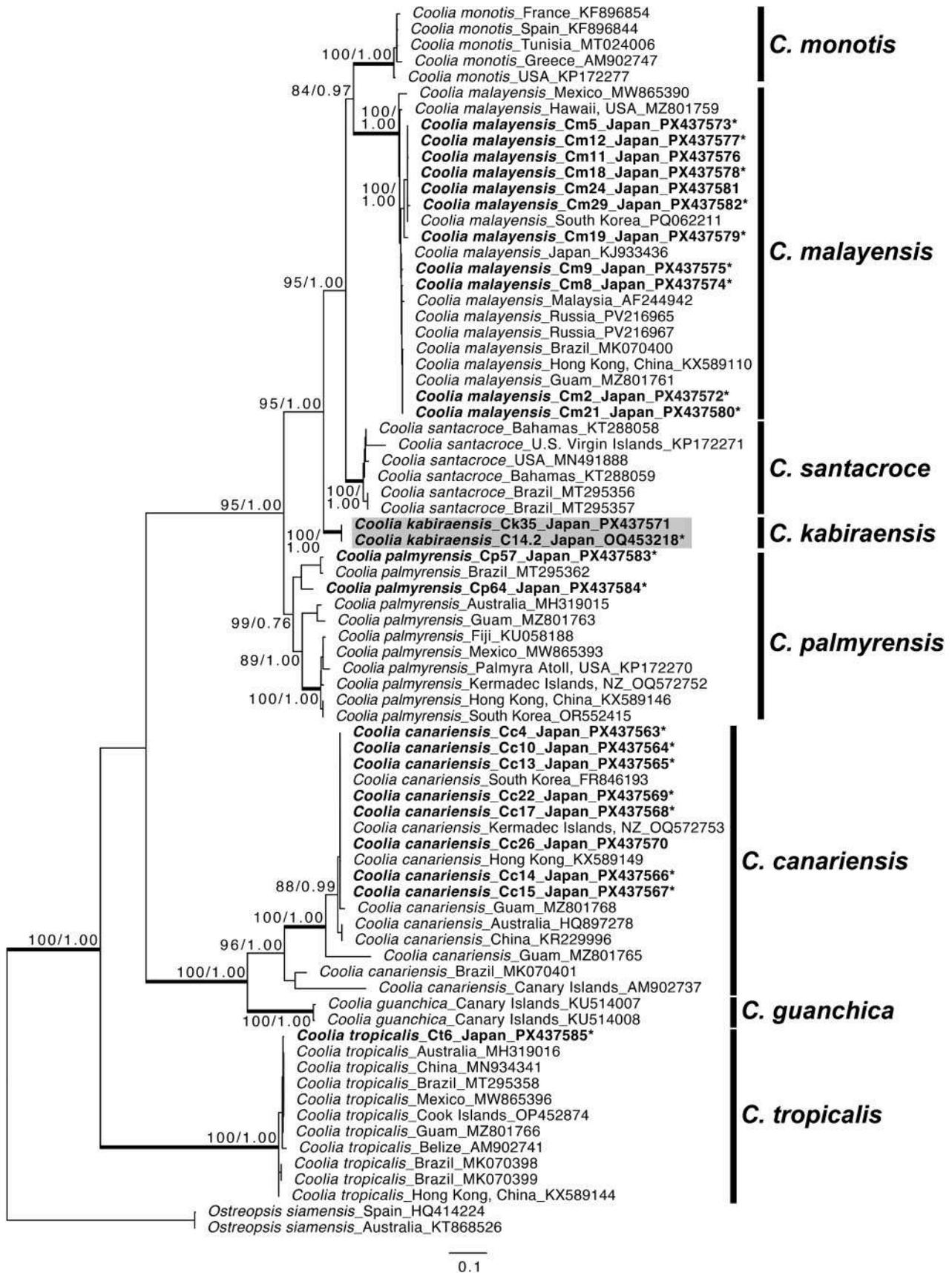


Fig. 5. Maximum likelihood (ML) phylogenetic tree of *Coolia* strains based on 28S (D1–D3) rDNA sequences. Numbers at nodes indicate ML bootstrap support (1000 pseudo replicates) and Bayesian Inferences (BI) posterior probabilities. Strains isolated in this study are shown in bold. Sequences of *Coolia kabiraensis* are highlighted in bold with a grey background. Asterisks (*) indicate strains used for *Artemia* toxicity bioassay.

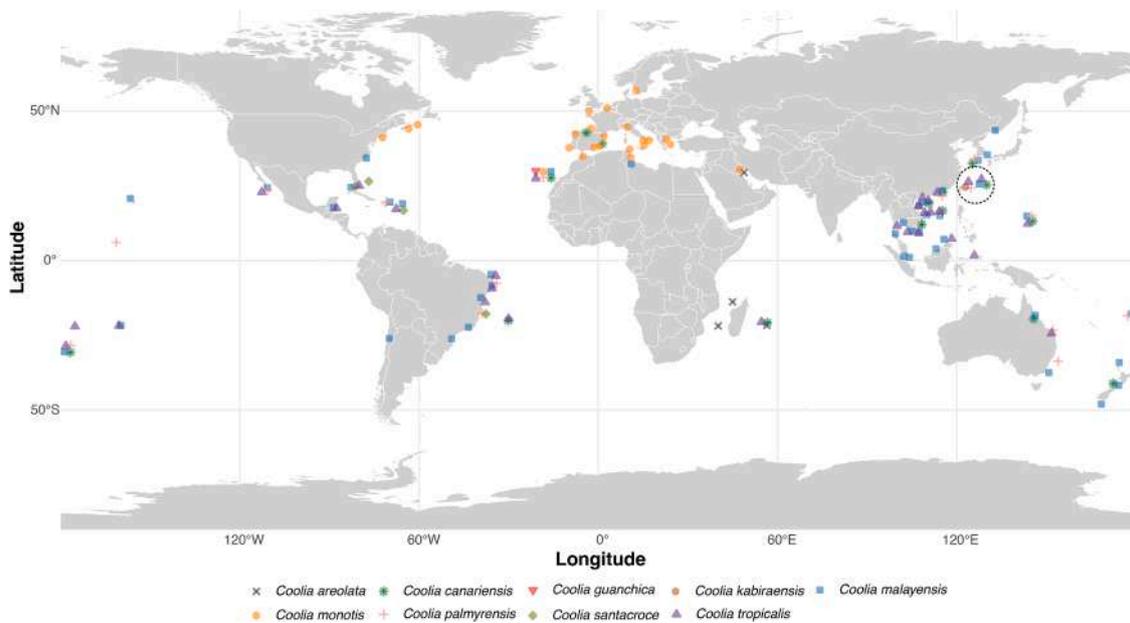


Fig. 6. Contemporary global distribution of *Coolia* species. Each point represents a reported collection locality of an isolate or strain, with unique colours and symbols for species. Sampling sites from the present study (Okinawa Prefecture, Japan) are marked with a black dashed circle. Overlapping records of the same species from the same general locality were visually collapsed to improve readability. Data were compiled from peer-reviewed literature, GenBank, grey literature, and culture collections (Table S3).

Table 1

Summary of the global distribution of *Coolia* based on published, georeferenced records. Regions reported, number of records, latitudinal ranges, and representative references are shown for each species. Full occurrence metadata are provided in Supplementary Table S3.

Species	Regions reported	Number of records	Latitudinal range	Key references
<i>Coolia areolata</i>	Western Indian Ocean, North-western Indian Ocean	4	21.9° S – 29.4° N	Al-Yamani and Saburova, 2010; Ten-Hage et al., 2000
<i>Coolia canariensis</i>	Western Atlantic, North-eastern Atlantic, Mediterranean, Western Indian Ocean, Indo-West Pacific, Australasia, North Pacific	50	41.2° S – 43.4° N	David et al., 2014; Fraga et al., 2008; Nascimento et al., 2019; Phua et al., 2021; Rhodes et al., 2023; Present study
<i>Coolia guanchica</i>	North-eastern Atlantic	5	28.1° N – 28.5° N	David et al., 2020
<i>Coolia kabiraensis</i>	Indo-West Pacific	2	25.5° N	Present study
<i>Coolia malayensis</i>	Western Atlantic, North-eastern Atlantic, Mediterranean, Indo-West Pacific, Australasia, Eastern Pacific, North Pacific	248	46.9° S – 43.1° N	Abdennadher et al., 2021; Leaw et al., 2010; Morquecho et al., 2022; Selina and Efimova, 2025; Uribe et al., 2023; Present study
<i>Coolia monotis</i>	North-western Atlantic, North-eastern Atlantic, Mediterranean, North-western Indian Ocean	103	28.3° N – 58.9° N	Aligizaki and Nikolaidis, 2006; Álvarez et al., 2022; David et al., 2014; Lewis et al., 2018; Meunier 1919; Penna et al., 2005
<i>Coolia palmyrensis</i>	Western Atlantic, North-eastern Atlantic, Indo-West Pacific, Australasia, Eastern Pacific, North Pacific	25	32.8° S – 33.2° N	Karafas et al., 2015; Larsson et al., 2019; Morquecho et al., 2022; Rhodes et al., 2023; Tibirićá et al., 2020; Present study
<i>Coolia santacroce</i>	Western Atlantic	10	18.0° S – 26.5° N	Karafas et al., 2015; Tibirićá et al., 2020
<i>Coolia tropicalis</i>	Western Atlantic, North-eastern Atlantic, Western Indian Ocean, Indo-West Pacific, Australasia, Eastern Pacific, North Pacific	62	29.3° S – 28.3° N	Faust 1995; Larsson et al., 2019; Morquecho et al., 2022; Rhodes et al., 2023; Present study

Considering historical records of *Coolia* from the Ryukyu Islands, dating back several decades, *C. tropicalis* was described from Ishigaki by Faust (1995), and Yasumoto et al. (1980) and Fukuyo (1981) reported what was then identified as *C. monotis*. The morphological description provided by Fukuyo (1981) is consistent with published descriptions of members of the *C. monotis* complex, and a *Coolia* culture from Hachijo-jima collected in 1984 and maintained by NIES further indicates early observations of this group in Japan. At that time, however, only a single taxon within the *C. monotis* complex was recognised. With current molecular data showing that true *C. monotis* is largely restricted to the Mediterranean and North Atlantic, historic Ryukyu records attributed to *C. monotis* are more likely referable to *C. malayensis* or another member

of the complex (Fig. 6; Larsson et al., 2019; Leaw et al., 2016). Our phylogenetic and biogeographic results support this interpretation and indicate that multiple *C. monotis* complex species occur in the region.

The closest genetic relatives to *C. kabiraensis* were *C. palmyrensis*, *C. santacroce*, *C. malayensis*, and *C. monotis*. While 28S rDNA divergence was slightly lower than some published *Coolia* species-level thresholds (e.g., *C. guanchica* in David et al., 2020; *C. palmyrensis* in Karafas et al., 2015), phylogenetic analyses placed *C. kabiraensis* between *C. palmyrensis* and *C. santacroce*. The 13–19% uncorrected p-distances observed here for *C. kabiraensis* are nevertheless broadly consistent with values separating recognized species of the *C. monotis* complex.

In contrast to 28S, ITS divergence was higher among the *C. monotis*

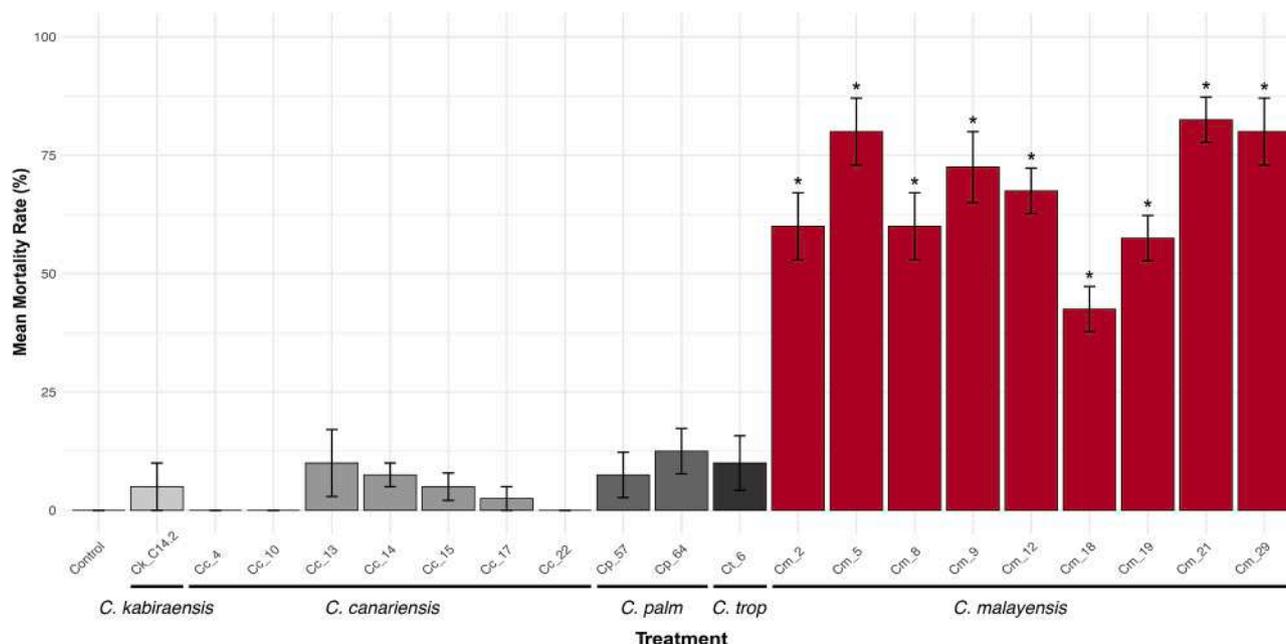


Fig. 7. Mortality of *Artemia* nauplii after 48 h of exposure to methanolic extracts from 20 *Coolia* strains. Both seawater and DMSO solvent controls resulted in zero mortality and are combined as 'Control' in the figure. Mortality is expressed as a percentage (mean \pm standard error, $n = 4$ wells per strain). Asterisks (*) indicate statistically significant differences compared to the control group ($p < 0.05$).

complex. The values observed for *C. kabiraensis* (17–32% uncorrected p-distance from other complex members) fall within the range of ITS divergences reported in prior species description studies (e.g., David et al., 2020; Karafas et al., 2015; Leaw et al., 2010). In our dataset, *C. monotis* ITS sequences showed similarly high divergence (15–31%) when compared with other members of the complex. For broader context among other Ostreopsidaceae, ITS divergences of 12–14% in *Ostreopsis* cf. *ovata* and *O. siamensis* have been interpreted as evidence of cryptic species lineages within those morphologically indistinguishable clades (Chomérat et al., 2020; Gu et al., 2022). The placement of *C. kabiraensis* in the 28S and ITS phylogenies, together with predicted ITS2 structural differences, provides consistent evidence supporting its distinctiveness relative to closely related species.

4.2. Global *Coolia* distribution

Coolia species are a widely distributed group of benthic dinoflagellates (Larsson et al., 2019; Leaw et al., 2016). *C. malayensis* is the most broadly distributed species, recorded from tropical and temperate zones in East and Southeast Asia, Oceania, and the Americas. *C. palmyrensis* and *C. tropicalis* are more confined to tropical and subtropical waters. Despite this broad distribution pattern, several cryptic or geographically restricted species are evident, including *C. santacroce*, *C. areolata*, *C. guanchica*, and the newly described *C. kabiraensis*, which is currently known only from Kabira Bay, Ishigaki Island.

In this study, we recorded four previously described *Coolia* species and identified one novel species, indicating relatively high *Coolia* diversity in a small geographic area of southern Japan. In Southeast Asia, *C. malayensis* and *C. tropicalis* are commonly reported (Ho and Nguyen, 2014; Leaw et al., 2010; Tawong et al., 2015). Additional species, such as *C. canariensis* and *C. palmyrensis*, have also been recorded in subtropical regions at similar latitudes to the Ryukyu Islands, including northern Vietnam, the Zhongsha Islands, Hong Kong, and Guam (Ho and Nguyen, 2014; Leung et al., 2017; Phua et al., 2021; Xie et al., 2022).

4.3. Ecotoxicity

Ryukyu-isolated strains of *C. kabiraensis*, *C. canariensis*, *C. palmyrensis*

and *C. tropicalis* showed low toxicity (<15% *Artemia* mortality), whereas all *C. malayensis* strains caused >40% mortality after 48 h. This pattern matches previous studies, including Leung et al. (2017) in Hong Kong and Phua et al. (2021) in Guam and Hawaii, where *C. malayensis* strains were more toxic than other local *Coolia* species. Such differences in toxicity among morphologically similar members of the *C. monotis* complex may provide additional evidence for species distinction. These toxicity levels are similar to other known toxic benthic species. For example, algal lysates of *Amphidinium carterae*, *Ostreopsis ovata*, *O. siamensis*, and *Prorocentrum lima* were lethal to brine shrimp, while *C. monotis* and *C. canariensis* were non-toxic (Laza-Martinez et al., 2011; Pagliara and Caroppo, 2012).

Contrastingly, Miralha et al. (2023) found that both live cells and cell-free media from *C. cf. canariensis* phylogroup II caused sub-lethal and lethal effects—and were more toxic than *C. malayensis*. This is notable because cell-free media of *Coolia* is generally considered less potent to brine shrimp than cell lysates or extracts like those used in our study (e.g., Leung et al., 2017), and *C. canariensis* has shown low toxicity in previous work (e.g., Leung et al., 2017; Phua et al., 2021), including our own.

Leung et al. (2017) reported that *C. canariensis* had no effect on brine shrimp but caused significant impacts on sea urchin larvae. These findings and those from Miralha et al. (2023) suggest that other *Coolia* species may be toxic via both intracellular (e.g., methanolic extracts, direct ingestion) and extracellular (e.g., cell filtrates, ambient seawater) compounds, and that observed toxicity depends on the test organism. Comparative studies using different exposure methods and biological targets (e.g., brine shrimp, marine invertebrate gametes and larvae, cell lines) are needed to clarify the mechanisms and ecological relevance of *Coolia* toxicity.

Although not directly quantified, we observed sub-lethal effects and abnormal swimming behaviour alongside mortality, suggesting that *Coolia* compounds may act through multiple mechanisms depending on concentration and exposure type. The specific pathways of *Coolia* toxicity remain unclear. Effects may result from known biotoxins such as yessotoxin or from unidentified compounds (Holmes et al., 1995; Phua et al., 2021; Wakeman et al., 2015). Further chemical characterisation of *Coolia* metabolites is needed to clarify their ecological roles and

potential contribution to benthic harmful algal events.

4.4. Climate change and potential range expansions

C. malayensis is notable for its broad geographic range, spanning tropical to temperate environments. Given its demonstrated toxicity and widespread distribution, it could be playing an underrecognized role in benthic harmful algal events. As ocean temperatures rise, this species could expand into higher-latitude temperate zones, increasing its impact in areas not previously affected. This possibility is supported by records of the closely related *C. monotis* at high latitudes, including Scandinavian waters (Álvarez et al., 2022) and a recent record of *C. malayensis* in east Russia coastal waters (Selina and Efimova, 2025), suggesting that some *Coolia* species tolerate lower temperatures and may shift poleward under future ocean warming scenarios. A recent study detected *C. palmyrensis* at more temperate latitudes off South Korea (Hyung et al., 2024). The authors used qPCR to track seasonal patterns and found that Korean populations of *C. palmyrensis* could overwinter. These findings further indicate that some *Coolia* species tolerate colder conditions than previously assumed and could persist or expand into higher latitudes under warming.

As ocean warming continues, some *Coolia* species are likely to expand their distributions poleward – similar to patterns observed and predicted for harmful planktonic algae (Hallegraeff, 2010; Hallegraeff et al., 2021; Rolton et al., 2022). If toxic species such as *C. malayensis* become more common and abundant in temperate waters, their effects on benthic ecosystems and food webs will require close attention. Future research should prioritise long-term monitoring of harmful benthic dinoflagellates in warming regions and experimental assessments of their thermal tolerance to better predict potential range shifts.

4.5. Seafood safety and ecotoxicology

Toxic *Coolia* species may affect benthic food webs by altering grazer behaviour or reducing grazer survival, similar to other toxic benthic dinoflagellates like *Gambierdiscus* and *Ostreopsis*. Bioactive compounds may also disrupt settlement of benthic invertebrates and accumulate in benthic-feeding fish and shellfish, posing risks to seafood safety—particularly in regions where *Coolia* is abundant. While *Coolia* has not been directly linked to major fish kills or human poisoning, its bioactive metabolites raise concerns about ecological effects and possible human exposure through seafood. These risks warrant further research, especially in warming regions.

4.6. Conclusion

This study provides a comprehensive assessment of *Coolia* diversity in the Ryukyu Islands, integrating morphological observations, molecular phylogenetics, and toxicity assays to identify five species, including one novel taxon. Our findings also contribute additional evidence toward understanding the ecological significance of *Coolia*, particularly regarding toxicity and global distribution patterns. Continued research combining morphological, molecular, toxicological, and ecological approaches will be essential for clarifying the roles of *Coolia* species in marine ecosystems and their potential contribution to benthic harmful algal bloom dynamics.

Author declaration

We confirm that:

1. The work described has not been published previously, except in the form of abstracts, preprints, published lectures, or academic theses.
2. The work is not under consideration for publication elsewhere.
3. Its publication is approved by all authors and, tacitly or explicitly, by the responsible authorities where the work was carried out.

4. All authors have significantly contributed to the manuscript and agree to be accountable for all aspects of the work.
5. The order of authors listed in the manuscript has been agreed by all.
6. The authors have disclosed any potential competing interests in the “Declaration of Interests” form.

CRediT authorship contribution statement

Shaun Cunningham: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Siratee Riewluang:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Aika Yamaguchi:** Writing – review & editing, Investigation, Data curation. **Anne Rolton:** Writing – review & editing, Visualization, Formal analysis. **Kevin C. Wakeman:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.2026.103075.

Data availability

Data will be made available on request.

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