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First report of *Coolia palmyrensis* in Korea: seasonal and spatial distribution of *C. palmyrensis* and *C. malayensis* in Korean coastal waters

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Distribution patterns of benthic dinoflagellates that are widely observed in tropical and temperate waters and have toxic potential are changing in response to ocean climate change. Although there have been no outbreaks associated with the genus *Coolia* affecting humans, it is crucial to understand their changing distribution and clearly identify the species in the study area to prepare for potential toxic events. In this study, five strains of *Coolia* species were isolated from macroalgae samples collected from Jeju Island and the eastern coastal waters of Korea. Through morphological and molecular analysis of these isolates, one strain was identified as *Coolia palmyrensis*, marking the first report of this species in Korea, and four strains as *C. malayensis*. One of the *C. malayensis* strains was isolated in Pohang on the eastern coast of Korea, where it had not been previously reported. From 2021 to 2023, monitoring of Jeju Island using a quantitative polymerase chain reaction (PCR) assay revealed that *C. palmyrensis* cells occurred mostly in autumn, with a maximum density of 242 cells g^{-1}, and overwintering populations were observed in 2023. However, *C. malayensis* cells were not observed in this area. Additionally, *C. malayensis* was observed in Pohang and Ulsan, located further north than Jeju Island with maximum cell densities of 537 and 201 cells g^{-1}, respectively. These data and our decade of monitoring experience confirmed the introduction and establishment of *C. palmyrensis* and habitat shift of *C. malayensis* in Korean coastal waters. This study provides advances for understanding of the relationships between climate-driven alterations and biogeographic distribution of these species.

KEYWORDS

benthic dinoflagellate, *Coolia palmyrensis*, *Coolia malayensis*, geographic distributions, ocean climate change

1 Introduction

Dinoflagellates—unicellular microeukaryotes—are crucial to complex marine ecosystems worldwide (Gómez, 2012; De Vargas et al., 2015). Less than 10% of dinoflagellate species inhabit benthic environments, where their characteristics, including morphology and biodiversity, have been adapted for symbiotic relationships with substrates, such as macroalgae (Rhodes et al., 2000; Hoppenrath et al., 2014). Research on benthic microalgae has focused on their potential to produce toxic compounds, resulting in harmful algal blooms (HAB) that threaten human health and aquatic environments (Ballantine et al., 1988; Granéli et al., 2002; Penna et al., 2005; Riobó et al., 2006). Since the 1970s, numerous studies have reported on the toxigenicity of benthic dinoflagellates: the genus *Gambierdiscus* produces ciguatera toxins, maitotoxin, and gambierol, whereas *Ostreopsis* and *Prorocentrum* produce palytoxin and okadaic acid, respectively (Yasumoto et al., 1987; Ten-Hage et al., 2000a; Cagide et al., 2011; Yogi et al., 2011; Parsons et al., 2012; Hoppenrath et al., 2013; Boente-Juncal et al., 2019). Owing to their potential toxicity affecting marine organisms and humans, numerous studies have focused on the early detection of these species and understanding their ecological characteristics, including artificial substrates (Tester et al., 2022), metabarcoding approach (Smith et al., 2017), and loop-mediated isothermal amplification (LAMP) assays (Lee et al., 2021).

The benthic dinoflagellate genus *Coolia* co-occurs with other epiphytic genera, such as *Ostreopsis* species, and exhibits a cosmopolitan distribution spanning tropical and temperate regions (Leaw et al., 2016). To date, no HAB events or outbreaks associated with *Coolia* species affecting humans have been reported, despite the observation of toxic molecules, such as yessotoxin analogs, 44-methylgambierone (44-MG), anhydrogambierone, and gambierone, in certain strains of *C. tropicalis* and *C. malayensis* (Holmes et al., 1995; Wakeman et al., 2015; Tibiriçá et al., 2020; Murray et al., 2024). However, it is necessary to monitor *Coolia* species regarding their distributions, substrate preferences, and toxic production. Alterations in existing environmental conditions in response to ocean climate change may be associated with an increase in their toxic potential (Wells et al., 2020; Anderson et al., 2021).

Molecular techniques in taxonomy enable accurate species identification, aiding in elucidating the distribution and biodiversity of *Coolia* species, despite their morphological similarity. The genus *Coolia* comprises eight species: *Coolia monotis* (Faust, 1992), *C. tropicalis* (Faust, 1995), *C. areolata* (Ten-Hage et al., 2000b), *C. canariensis* (Fraga et al., 2008), *C. malayensis* (Leaw et al., 2010), *C. santacroce*, *C. palmyrensis* (Karafas et al., 2015), and *C. guanchica* (David et al., 2020). Before applying molecular data to confirm these taxa, morphologically similar species were occasionally misidentified because of their phenotypic plasticity and the presence of cryptic or pseudo-cryptic features within the species (Leaw et al., 2016). However, by combining morphological and molecular analyses, certain strains previously classified as *C. monotis* have now been reassigned to *C. malayensis*, *C. santacroce*, and *C. palmyrensis* (Karafas et al., 2015). Although there are no molecular data for *C. areolata*, its cells can be distinguished based on their morphological characteristics (Ten-Hage et al., 2000b). Recently, *C. canariensis*, which exhibits cryptic diversity, has been divided into four clades in phylogenetic relationships, known as the *Coolia canariensis* species complex. The geographic distribution of each phylogroup has been studied in distinct intraspecific taxa (Miralha et al., 2023).

Based on the literature that provides insights into both morphological and molecular data of isolated strains, *C. malayensis* is widely distributed in temperate and tropical regions. In contrast, *C. santacroce*, *C. palmyrensis*, *C. tropicalis*, and *C. canariensis* are observed in tropical waters of both the Atlantic and Pacific oceans, though *C. canariensis* is primarily found in the subtropical Canary Islands (Leaw et al., 2016). However, the distribution patterns of benthic dinoflagellates, such as *Ostreopsis*, are changing in response to climate change (Wells et al., 2020; Verma et al., 2023). Similarly, *Coolia* species known to inhabit tropical waters have been observed in the South Atlantic and Pacific waters. For example, *C. tropicalis* and *C. canariensis* have been observed on Trindade Island, Brazil (Nascimento et al., 2019), *C. tropicalis* and *C. palmyrensis* on Heron Island, Australia (Larsson et al., 2019), and *C. santacroce* and *C. palmyrensis* on Bahia, the northeastern Brazilian coast (Tibiriçá et al., 2020). Additionally, *C. monotis* has been observed on Rhode Island, USA, northwestern Atlantic waters, and Nova Scotia, Canada, although this species has been traditionally recorded only in the Mediterranean Sea (Lewis et al., 2018). Since 2011, benthic dinoflagellates, including *Coolia*, have been reported in Korea and identified at the genus level based on morphological analysis without molecular support (Kim et al., 2011; Baek, 2012; Shah et al., 2013). Subsequently, two *Coolia* species (*C. canariensis* and *C. malayensis*) were observed in the waters of Jeju Island that were confirmed through their morphological and molecular data (Jeong et al., 2012). However, the occurrence and distribution patterns of *C. palmyrensis*, which has not been previously observed in Korean coastal waters, and *C. malayensis* remain unexplored in Korea.

In this study, we performed a comprehensive sampling of benthic dinoflagellates collected from macroalgae and isolated one strain of *C. palmyrensis* and four strains of *C. malayensis* from Jeju Island and the eastern coastal waters of Korea. These strains were examined using light microscopy, scanning electron microscopy, and molecular data derived from the D1-D3 regions of large subunit ribosomal DNA (LSU rDNA) and internal transcribed spacer (ITS rDNA) regions for taxonomical analysis. To better understand the geographic distribution patterns of Korean *Coolia* species, we monitored the occurrence of *C. palmyrensis* and *C. malayensis* in environmental samples collected from macroalgae on Jeju Island, Korea, from 2021 to 2023, using the species-specific quantitative polymerase chain reaction (qPCR).

2 Materials and methods

2.1 Sampling, isolation, and establishment of strain

From 2021 to 2023, seasonal sampling was conducted 4 to 5 times per year at four locations on Jeju Island, Korea—Dodu (33°

Abbreviations: LAMP, loop-mediated isothermal amplification; APC, apical pore complex.

30'38.9"N 126°28'46.0"E), Seogwi (33°14'21.0"N 126°34'10.9"E), Seongsan (33°27'36.9"N 126°56'02.0"E), and Sinchang (33° 20'57.7"N 126°10'45.7"E)—representing the north, south, east, and west of the island (Figure 1). The number of sampling events varied slightly due to weather conditions and the need for additional surveys during periods of heightened benthic dinoflagellate activity. Along the eastern coast of Korea, additional samples were collected sporadically from two sites, Pohang (36°08'32.0"N and 129° 23'48.2"E) and Ulsan (35°36'46.2"N and 129°27'29.0"E), during June and August 2021, and June and October 2023. At each site, 2 to 4 macroalgal samples were collected, depending on the weather conditions at the time of sampling. Physical parameters, such as seawater temperature and salinity, were recorded at each sampling site using a YSI Professional Plus (Xylem, Inc., Rye Brook, USA).

In this study, various species of macroalgae were collected and analyzed, representing three major groups: red algae (including *Corallina pilulifera*, *Grateloupia cornea*), brown algae (including *Marginisporum crassissimum*, *Sargassum fusiforme*), and green algae (including *Ulva australis*, *Ulva linza*). At each site, macroalgal samples were collected through scuba diving using clean sealable plastic bags. Subsequently, they were transferred to polycarbonate (PC) bottles and gently shaken to detach benthic dinoflagellate cells from the substrates. The mixed seawater samples were filtered through glass microfiber filters with a 1.2 µm pore size (GF/C; Whatman® Inc., Maidstone, United Kingdom) to obtain environmental DNA (eDNA). All samples were frozen on dry ice, delivered to the laboratory, and stored at -80°C until DNA extraction. The final volumes of seawater collected for each macroalgal species in the plastic bag and the fresh weights of each macroalgal species were measured to quantify the cell concentrations using qPCR. For the isolation of microalgae strains, 300 mL of mixed seawater samples were collected and transported live to the laboratory.

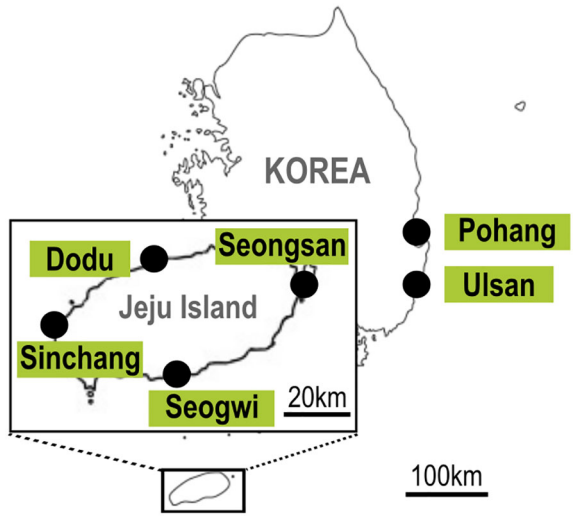

FIGURE 1
Location maps of sampling sites on Jeju Island covering the north, south, east, and west: Dodu, Seogwi, Seongsan, and Sinchang. Further sampling sites include Pohang and Ulsan in the eastern coastal waters of Korea.

To establish the clonal strains of *Coolia*, the live sample transported to the laboratory was placed in a six-well plate, and single-cell isolation was performed under a dissecting microscope (SZX10; Olympus, Tokyo, Japan). After single-cell isolations with 5 washings were established, the cells were cultured at 21°C with continuous illumination of 65 µmol m^{-2} s^{-1} photon irradiance in autoclaved filtered seawater containing f4-si media (Guillard and Ryther, 1962). After sufficient growth, the dense cultures were transferred to 50, 250, and 500 mL PC bottles, established as monoclonal culture strains, and subcultured monthly. The *C. palmyrensis* strain was used for morphological and molecular identification, including phylogenetic relationships, while all four strains of *C. malayensis* were taxonomically identified for molecular phylogeny.

2.2 Morphology

The morphology of living cells of *C. palmyrensis* or *C. malayensis* was observed using an inverted microscope (Olympus BX53) for drawing the cell shape and thecal plate pattern. The live cell dimensions were measured using a digital camera (Olympus DP73; Tokyo, Japan). For scanning electron microscopy (FE-SEM, Sigma 500/VP; Carl Zeiss, Oberkochen, Germany), culture samples (20 mL) containing approximately 3,000 cells mL^{-1} of *C. palmyrensis* or *C. malayensis* were fixed in seawater with a final concentration of 2% (v/v) glutaraldehyde for 1 h. The fixed cells were collected on a PC membrane filter (pore size 5 µm) without additional pressure and rinsed thrice with distilled water to remove salts. Dehydration was performed using a graded ethanol series (10, 30, 50, 70, 90, and 100% ethanol, followed by two 100% ethanol steps), and the final drying was performed using a critical point dryer (EM CPD300; Leica, Wetzlar, Germany) (Kang et al., 2010). The anteroposterior (AP) length, dorsoventral (DV) length, and width of *C. palmyrensis* or *C. malayensis* cells were measured using SEM micrographs, selecting only those images demonstrating the complete shape and size of each plate (viewed from directly above).

2.3 DNA extraction, PCR, and sequencing

Each monoclonal culture (30 mL) of the five *Coolia* strains was centrifuged (3000 × *g*, 10 min), harvested as cell pellets, and frozen at -80°C until DNA extraction. The preserved cell pellets were homogenized using Bel-Art® Disposable Micro Pestles, and genomic DNA (gDNA) was extracted using a GeneAll® Exgene™ Plant SV mini kit (GeneAll Biotechnology Co., Ltd., Korea), following the manufacturer's protocol.

For species identification based on ribosomal DNA (rDNA) sequence analysis, the ITS region was amplified using the primer pairs ITSF2-LSUB and ITSF2-ITSR2 (Litaker et al., 2003) obtained from the extracted gDNA samples. Amplification of the D1-D3 region of LSU rDNA was performed using the primer pairs D1R-D3B (Nunn et al., 1996) and D2CF-1256R (Momigliano et al., 2013). The PCR mixture was 50 µL, containing 5 µL of 10× F-Star Taq Reaction Buffer, 38.75 µL of UltraPure™ DNAse/RNAse-Free Distilled Water (Invitrogen, Carlsbad, CA, USA), 1 µL of 10 mM deoxyribonucleotide

triphosphate (dNTPs), 0.25 μL of 5 U μL^{-1} BioFACT™ F-Star Taq DNA Polymerase (BioFACT Co., Ltd., Daejeon, Korea), 0.02 μM of both forward and reverse primers, and 3 μL of template DNA. PCR was performed using the Eppendorf Master Cycler PCR machine (Eppendorf, Hamburg, Germany) under the following thermocycling conditions for the primer pair ITSF2-LSUB: pre-denaturation at 94°C for 5 min; followed by 40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 1 min; with a final extension at 72°C for 10 min. The thermocycling conditions for ITSF2-ITSR2, D1R-D3B, and D2CF-1256R were pre-denaturation at 94°C for 5 min, followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. All PCR products were purified using an AccuPrep PCR Purification Kit (BIONEER) and sent to Bionics (Bionics Co., Ltd., Daejeon, Korea) for Sanger sequencing. The results were aligned using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI).

2.4 Phylogenetic analysis

The analyzed sequences of five isolates for each gene region were aligned using the ClustalW algorithm with other sequences of the *Coolia* relatives obtained from the public GenBank database (http://www.ncbi.nlm.nih.gov). In the ITS region, 46 sequences were compared, including the five isolates from this study and four outgroups. For the D1-D3 region of LSU rDNA, 65 sequences were taxonomically analyzed, including one outgroup. After alignment, all data were manually trimmed, and misalignments were inspected using the SILVA alignment and software package ARB project 6.0.6 (Pruesse et al., 2012). A Maximum Likelihood (ML) analysis was performed using MEGA7, calculating the bootstrap values for both ITS and D1-D3 LSU regions with 1000 replicates based on the Jukes-Cantor model to assess statistical reliability (Kumar et al., 2016). Phylogenetic reconstruction for sequence alignment was inferred using Bayesian inference (BI) by estimating evolutionary distances using BEAST 1.10.4 (Drummond et al., 2012). Consensus trees were generated and visualized using the TreeAnnotator 1.10.4 (Rambaut and Drummond, 2013) and Figtree 1.4.5 (Rambaut, 2009), respectively. The BI tree displayed Bayesian posterior probability values, which indicated statistical reliability.

2.5 TaqMan-based qPCR assay

Based on the sequences acquired from the isolates *C. palmyrensis* and *C. malayensis*, the species-specific primer and Taqman probe sets were designed using Beacon Designer 8.21 and Primer3 (version 0.4.0) software (http://bioinfo.ut.ee/primer3-0.4.0/), targeting the D1-D3 region of the LSU rDNA and ITS region, respectively (Table 1). It was confirmed that all the delta G values of self-dimers, hairpins, and cross-dimers were weaker (more positive) than -2.4 kcal mol^{-1} from the designed primer and probe sets. The specificity of the primer sequences was confirmed using BLAST at the NCBI. The qPCR assay for cross-reactivity testing with other dinoflagellate species was assessed using gDNA samples extracted from clonal cultures of *C. palmyrensis* JSGP strain, four isolates of *C. malayensis*, and other established laboratory species using the GeneAll® Exgene™ Plant SV mini kit as described above (Table 2). For qPCR assays, the presence of *C. palmyrensis* and *C. malayensis* was quantified in samples collected from macroalgae on Jeju Island from 2021 to 2023. To assess the distribution of the benthic dinoflagellate *Coolia* in the East Sea of Korea, we analyzed the occurrence of these two species using qPCR at Pohang and Ulsan in the eastern coastal waters of Korea during June and August 2021 and June and October 2023. The qPCR mixtures contained 10 μL of qPCRBIO Probe Mix no-ROX (2×) (PCR Biosystems, London, England), 1 μL of 10 μM primer pairs, 0.5 μL of 10 μM TaqMan probe, 4.5 μL of UltraPure™ DNAse/RNAse-Free Distilled Water (Invitrogen), and 3 μL of template DNA in a final volume of 20 μL. Thermal cycling of the qPCR assay was performed using a PCRmax Eco 48 Real-time PCR System (Staffordshire, UK) under the following conditions: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 10 s and extension at 57°C (*C. palmyrensis*) or 54.5°C (*C. malayensis*) for 30 s, in the presence of no template control. Each standard curve of *C. palmyrensis* and *C. malayensis* for the qPCR assay was determined using the extracted gDNA based on a specific number of cells (40000, 20000, 4000, 2000, 400, and 40). The coefficient of determination (R^2) and amplification efficiency for each assay were calculated from the standard curve-derived linear regression curves. For the assay performed using 10-fold serially diluted DNA extracts from each species, the limit of detection for both *C. palmyrensis* and *C. malayensis* was below 0.4 cells per reaction.

TABLE 1 Sequences of primers and probes for the quantitative polymerase chain reaction (qPCR) assay targeting *Coolia palmyrensis* and *Coolia malayensis*.

Target species	Primer name	Sequences (5′ > 3′)	Target	Product size	Reference
Coolia palmyrensis	Forward primer: CpF	GCCAAGGAATGTATCTTGAA	D1-D3 LSU rDNA	125 bp	In this study
	Reverse primer: CpR	TGTTTAATTCACATGGGCAT			
	Taqman probe: CpP	CGCTTGAACTGCACCGTTGGAG			
Coolia malayensis	Forward primer: CmF	GCGGAAGGATCATTTGTGTT	ITS	142 bp	In this study
	Reverse primer: CmR	TCATTGCCAAACACACACAC			
	Taqman probe: CmP	CCATCAGTTTATAACCAAAACCATACCATAT			

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TABLE 2 Strains used for validating the specificity of *Coolia palmyrensis* and *Coolia malayensis* quantitative polymerase chain reaction (qPCR) assay.

Species name	Strain	Origin	Result for the *C. palmyrensis* qPCR assay	Result for the *C. malayensis* qPCR assay
Coolia palmyrensis	JSGP	Seogwi, Jeju Island	+/+	-/-
Coolia malayensis	EPH210527	Pohang	-/-	+/+
Coolia malayensis	JSC201014	Sinchang, Jeju Island	-/-	+/+
Coolia malayensis	JSGP201118	Seogwi, Jeju Island	-/-	+/+
Coolia malayensis	JJJ210831	Dodu, Jeju Island	-/-	+/+
Alexandrium catenella	SDDP190523	Dadaepo, Busan	-/-	-/-
Alexandrium pacificum	MS	Masan	-/-	-/-
Ceratium furca	JSS	Jinhae	-/-	-/-
Fibrocapsa japonica	CCMP1661	Melbourne, Victora, Australia	-/-	-/-
Gambierdiscus jejuensis	JJJ210127	Dodu, Jeju Island	-/-	-/-
Gymnodinium catenatum	SGJ200924	Gijang, Busan	-/-	-/-
Gymnodinium instriatum	SJH230621	Jinhae	-/-	-/-
Margalefidinium polykrikoides	SWD150911	Wando	-/-	-/-
Ostreopsis cf. ovata	YD	Gimyeong, Jeju Island	-/-	-/-
Prorocentrum micans	EDH210722	Donghae	-/-	-/-
Scrippsiella trochoidea	US	Ulsan	-/-	-/-
Symbiodinium voratum	JSS201116	Seongsan, Jeju Island	-/-	-/-
Tetraselmis jejuensis	JJJ190616	Yongduam, Jeju Island	-/-	-/-
Torquentidium flavescens	SGJ2009224	Gijang, Busan	-/-	-/-

Results are demonstrated in either positive (+) or negative (-) detection as duplicate reactions.

3 Results

3.1 Environmental conditions of isolated strains

Five monoclonal culture strains of the *Coolia* species were established in the laboratory: one strain of *Coolia palmyrensis* (JSGP) and four strains of *C. malayensis* (EPH210527, JSC201014, JSGP201118, and JJJ210831). The JSGP strain of *C. palmyrensis* was isolated from Seogwi on Jeju Island, Korea, where the seawater temperature was 21.4°C and salinity was 32.80 psu (Table 3). Three strains of *C. malayensis* (JSGP201118, JJJ210831, and JSC201014) were isolated from Seogwi, Dodu, and Shinchang sites on Jeju Island, with water temperatures and salinities as follows: 21.4°C and 32.80 psu, 27.3°C and 30.79 psu, and 22.7°C and 33.54 psu, respectively. Another strain, EPH210527, was isolated from Pohang on the eastern coast of Korea, with a seawater temperature of 15.8°C and salinity of 33.42 psu. The surface seawater temperature and salinity data in Table 4 summarize the seasonal variations observed at Jeju Island from 2021 to 2023. For

TABLE 3 Details of *Coolia* strains isolated in this study.

Species name	Strain	Origin	Temperature (°C)	Salinity (psu)	Accession number	
					ITS	LSU
Coolia palmyrensis	JSGP	Seogwi, Jeju Island	21.4	32.8	OR552419	OR552415
Coolia malayensis	EPH210527	Pohang	15.8	33.42	PQ060260	PQ062211
Coolia malayensis	JSC201014	Sinchang, Jeju Island	22.7	33.54	PQ060261	PQ062212
Coolia malayensis	JSGP201118	Seogwi, Jeju Island	21.4	32.8	OL966569	OL982547
Coolia malayensis	JJJ210831	Dodu, Jeju Island	27.3	30.79	PQ060359	PQ062213

TABLE 4 Seasonal variations in surface seawater temperature (°C) and salinity at Jeju Island, Korea, from 2021 to 2023.

Year	Season	Jeju Island	
		Temp. (°C)	Sal.
2021	Spring	17.5 ± 1.0	33.6 ± 1.4
	Summer	25.6 ± 2.4	29.4 ± 3.8
	Autumn	21.0 ± 1.4	32.9 ± 1.3
	Winter	14.9 ± 1.2	33.8 ± 0.9
2022	Spring	17.4 ± 0.8	32.8 ± 2.4
	Summer	23.8 ± 2.9	32.2 ± 1.3
	Autumn	22.6 ± 1.5	30.8 ± 2.2
	Winter	14.5 ± 0.8	33.5 ± 0.3
2023	Spring	17.3 ± 0.5	34.1 ± 0.8
	Summer	24.2 ± 3.4	29.9 ± 2.8
	Autumn	22.3 ± 1.1	32.0 ± 0.9
	Winter	15.0 ± 0.6	31.6 ± 1.8

The data represent the mean ± standard deviation for the four seasons: spring, summer, autumn, and winter.

the stations in Pohang and Ulsan on the eastern coast of Korea, data were collected in June and August in 2021, and June and October in 2023 (Table 5).

3.2 Morphology of the Korean strain of *Cooila palmyrensis*

The cells were nearly spherical or ovoidal in both apical and antapical views and slightly compressed in the lateral view (Figures 2A–F). The hypotheca was slightly larger or similar in size to that of the epitheca. The AP length of the cells ranged from 17.4–30.1 µm, and the width ranged from 18.6–29.4 µm. The DV depth ranged from 19.5–26.0 µm. The thecal surface was generally smooth. The cingulum, which was indented in the equatorial region, encircled the transverse axis of the cell. The sulcus was deeply indented on the ventral side of the hypotheca, but did not extend to the bottom (Figure 2D).

The thecal plate formula was apical pore complex (APC), 3', 7", 5"', and 2"". The epitheca consisted of three apical plates (1'–3'), APC, and seven pre-cingular plates (1"–7"). The pentagonal plate 6" was the largest in the epitheca, followed by the hexagonal plate 1'. The 3' plate was pentagonal (Figures 2A, B, E). The pentagonal plate 7" ranged from 3.8–9.1 µm in height and 5.5–8.3 µm in width, with a width-to-height ratio of 1.2 to 2.1. The hypotheca was composed of five post-cingular (1"'–5"') and two antapical (1""–2"") plates. Plates 3"' and 4"' occupied the majority of the hypotheca, with plate 3"' being the widest, often covering more than half of the hypotheca surface area. Plates 2"' and 5"' followed in size. Plate 2"' was adjacent to plates 1"', 3"', and 1"" (Figures 2C, F).

TABLE 5 Surface seawater temperature (°C) and salinity data collected from Pohang (PH) and Ulsan (US) on the eastern coast of Korea during June and August in 2021, and June and October in 2023.

Year	Month	Pohang (PH)		Ulsan (US)	
		Temp. (°C)	Sal.	Temp. (°C)	Sal.
2021	June	25.0	33.5	21.9	33.5
	August	26.1	30.2	25.2	31.3
2023	June	23.1	34.1	21.1	34.2
	October	22.2	31.0	22.1	31.8

3.3 Morphology of the Korean strain of *Cooila malayensis*

In this study, the morphological characteristics of *C. malayensis* isolated in this study were identical to those reported for the first time in Korea (Jeong et al., 2012). The cell shape was nearly spherical, with a slight depression in the center when viewed ventrally. From the lateral view, it appeared slightly tilted and ovoid, resembling a pointed teardrop when viewed apically (Figures 3A–D). The hypotheca was similar in size or slightly larger than that of the epitheca. The AP cell length ranged from 20.8–27.0 µm, width ranged from 22.4–27.9 µm, and DV thickness ranged from 24.6–30.3 µm. Circular pores were randomly distributed across the thecal surface. The sulcus was deeply indented in the ventral hypotheca, but did not extend to the bottom.

The thecal plate formula comprised APC, 3', 7", 5"', and 2"". The epitheca consisted of three apical plates (1'–3'), APC, and seven pre-singular plates (1"–7") (Figure 3C). In the side view, it appeared as a straight slit, slightly covered by plates 1', 2', and 3', with an APC length of 4.6–5.7 µm (Figure 3B). Plates 1" to 7" were arranged counterclockwise around the sulcal axis. The hypotheca consisted of five post-cingular plates (1"'–5"') and two antapical (1""–2"") plates. Plates 3"' and 4"' occupied the majority of the hypotheca. Plate 1"" was on the right side and plate 2"" on the posterior part of the sulcus. The thecal surface contained numerous pores (Figure 3D).

3.4 Phylogeny of *Coolia* species

The molecular taxonomy of *Coolia* strains isolated from Jeju Island and the East Sea of Korea was analyzed using Bayesian phylogenies of ITS regions and D1-D3 LSU rDNA sequences. This included the strains from this study and additional reference sequences from GenBank. In the ITS trees, four strains—JSGP201118, JJJ210831, and JSC201014, isolated from Jeju Island, and EPH210527 from Pohang in the East Sea—were placed within the phyletic clade of *C. malayensis*, with highly supported statistical values of ML and posterior probabilities of BI at node (93/1.00) (Figure 4). The strain EPH210527 diverged from the other three statins within *C. malayensis*, with ML and BI values of 78 and 1.00, respectively, at node. However, the strain JSGP isolated in this study was clustered with the *C. palmyrensis* and supported by ML and BI values of 99 and 1.00, respectively. In the LSU trees, the four strains of *Coolia* were grouped into the phyletic clade of *C. malayensis* with

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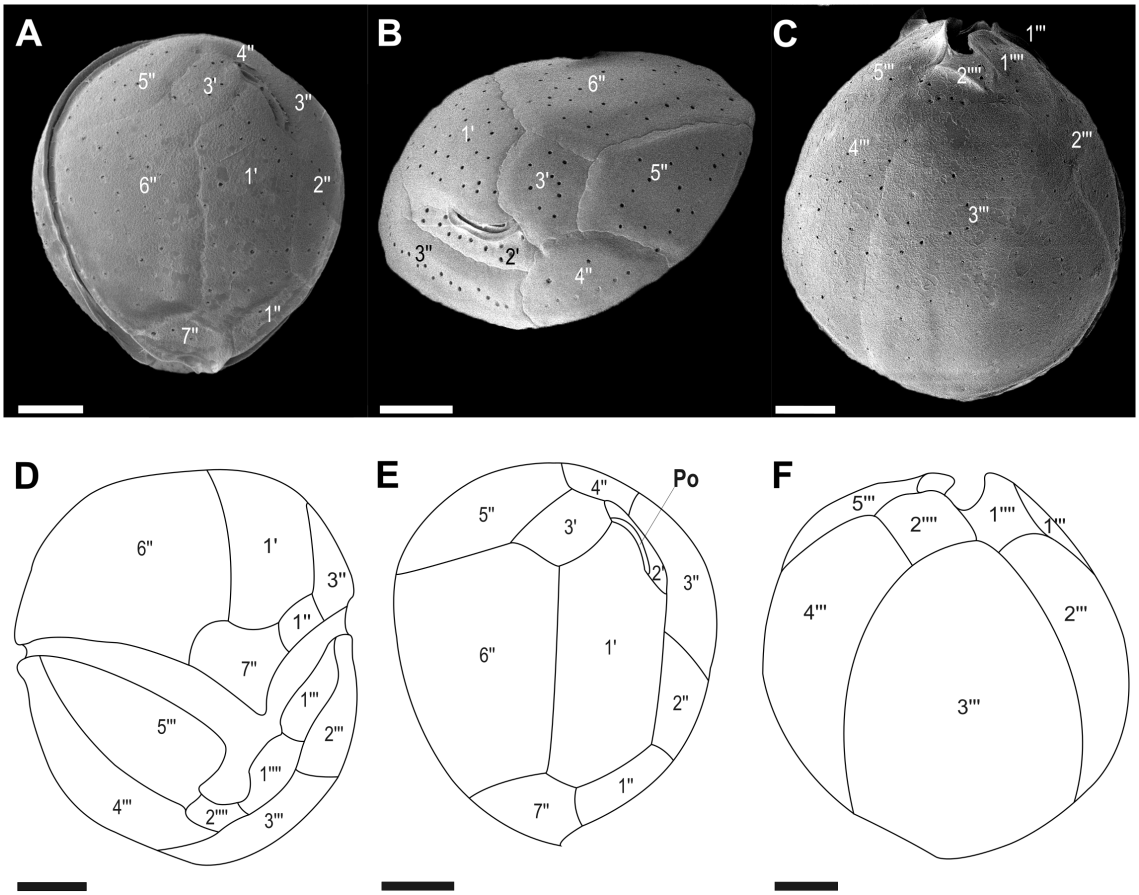

FIGURE 2
Micrographs of *Coolia palmyrensis* cells obtained using scanning electron microscopy (SEM): **(A, B)** apical views and **(C)** antapical view. Line drawings of **(D)** ventral view, **(E)** apical view, and **(F)** antapical view. Scale bar = 5 μm.

high ML and BI values of 99 and 1.00, respectively (Figure 5), whereas JSGP was clustered with the *C. palmyrensis*, supported by ML and BI values of 94 and 1.00, respectively.

3.5 Distributions of *Coolia* species

For the qPCR assay, a cell-based standard curve was established based on a linear slope between the log cell numbers of *C. palmyrensis* and cycle threshold (Ct) values, with R^2 and amplification efficiency of 0.94 and 85%, respectively (Figure 6A). Additionally, the qPCR assays using primers targeting the ITS region of the rDNA of *Coolia malayensis* amplified the target species, with no detection of other *Coolia* species and dinoflagellates. The cell-based standard curve, plotted using a log-linear portion, exhibited good R^2 and amplification efficiency of 0.98 and 108%, respectively (Figure 6B).

We analyzed 154 samples collected across four locations (north, south, east, and west) on Jeju Island for the presence of *C. palmyrensis* using qPCR between 2021 and 2023. In 2021, positive detections were observed on diverse macroalgal species between the summer and autumn. During summer, low cell concentrations (2 cells g^{-1} fresh weight (FW) macroalgae) were detected only in the north, whereas in autumn, the cells were distributed throughout the region in all four directions (Figure 7A). The maximum abundances were 11 and 9 cells g^{-1} FW macroalgae in the north and south, respectively. However, significantly higher concentrations were recorded in the west and east, with 107 and 242 cells g^{-1} FW macroalgae, respectively. In 2022, the cells were detected in the north during spring at a density of 68 cells g^{-1} FW macroalgae, and in both the north and south during autumn at concentrations < 10 cells g^{-1} FW macroalgae. From spring to autumn of 2023, the cells were positively identified in the northern (Dodu) and eastern (Seongsan) regions. Concentrations were < 10 cells g^{-1} FW macroalgae at all points except in the north during summer, when the maximum cell density was 12 cells g^{-1} FW macroalgae. The cells survived during winter in the south (Seogwi) of Jeju Island at a density of 28 cells g^{-1} FW macroalgae on the macroalga *Plocamium uncinatum*. In contrast, *C. malayensis* was not detected in any of the samples collected from Jeju Island during the study period (Figure 7B).

In June, cell densities on four macroalgal species (*Ulva linza*, *Sargassum coreanum*, *Sargassum miyabei*, and *Ulva australis*) collected in Pohang were 48, 312, 38, and 537 cells g^{-1} FW macroalgae, respectively (Figure 8, June #1–#4). In Ulsan, the cell density of the macroalgae *Callophyllis adnata* was 201 cells g^{-1} FW macroalgae, whereas no cells were identified in August. *Coolia palmyrensis* was detected with a maximum abundance of 13 cells

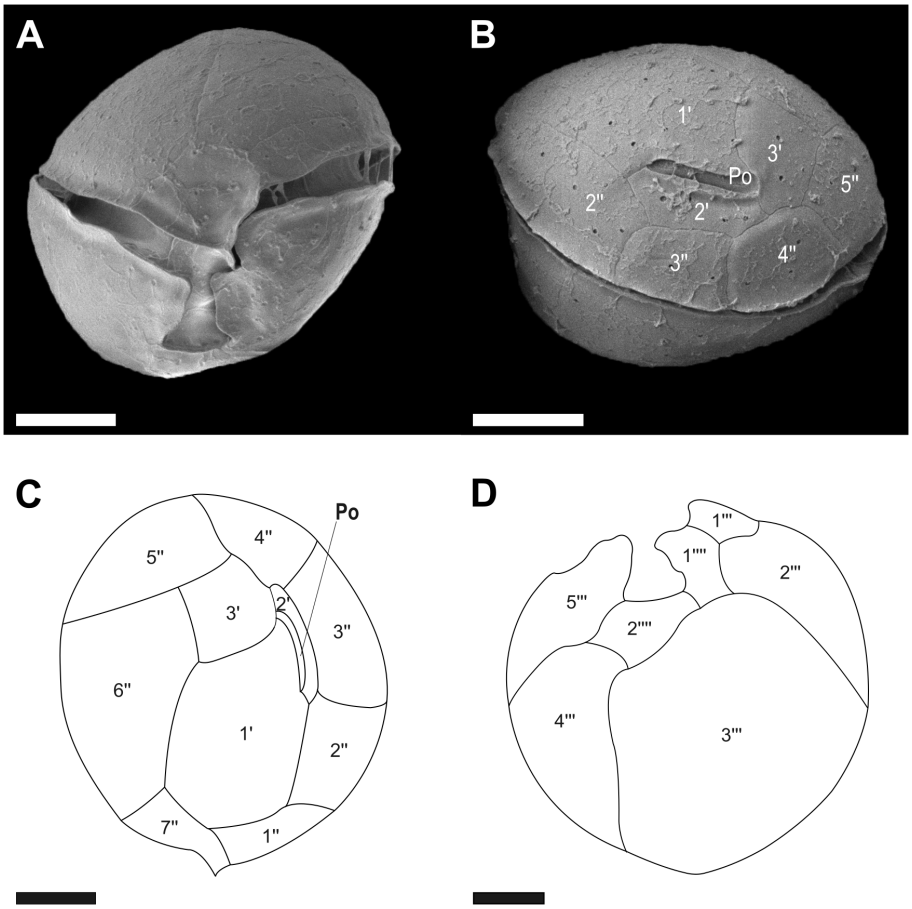

FIGURE 3
Micrographs of *Coolia malayensis* cells obtained using scanning electron microscopy (SEM): **(A)** ventral view and **(B)** dorsal view. Line drawings of **(C)** apical view and **(D)** antapical view. Scale bar = 5 µm.

g^{-1} FW macroalgae on the macroalgae *Gracilariales* in Pohang, and 8 cells g^{-1} FW macroalgae on the macroalgae *Corallina pilulifera* in Ulsan in August, with no cells detected in June. In 2023, *C. palmyrensis* cells were at a density of 1 cell g^{-1} FW macroalgae on *Sargassum* macroalgae in Pohang in June, whereas *C. malayensis* cells were at a density of 11 cells g^{-1} FW on *Plocamium uncinatum* in similar locations and months.

4 Discussion

Species within the genus *Coolia* are predominantly found in tropical and temperate marine regions. To date, eight species have been reported: *C. areolata*, *C. canariensis*, *C. guanchica*, *C. malayensis*, *C. monotis*, *C. palmyrensis*, *C. santacroce*, and *C. tropicalis* (David et al., 2020; Faust, 1995; Fraga et al., 2008; Karafas et al., 2015; Leaw et al., 2010; Meunier, 1919; Ten-Hage et al., 2000b). *C. palmyrensis* was initially reported by Karafas et al. (2015) from Palmyra Atoll in the middle of the Pacific Ocean, near the equator. The plate formula of *C. palmyrensis* isolated from Jeju, Korea, was identical to that of the original report of this species. However, in this study, the 3' plate was pentagonal, whereas Karafas et al. (2015) described it as either pentagonal or hexagonal. In contrast, the 3' plate of *C. malayensis* was quadrangular and did not contact the 5" plate in its original description (Leaw et al., 2016), which differentiates it from *C. palmyrensis*, whose 3' plate is pentagonal and contacts the 5" plate. However, subsequent studies on other *C. malayensis* populations have demonstrated that the 3' plate can also be pentagonal and contact the 5" plate, similar to *C. palmyrensis*, indicating variability in the 3' plate shape within *C. malayensis* populations (Jeong et al., 2012; de Queiroz Mendes et al., 2019). Because *C. areolata*, *C. canariensis*, *C. malayensis*, *C. monotis*, and *C. palmyrensis* exhibit similar morphological characteristics, such as cell shape, cell size, and plate arrangement, distinguishing among these species is crucial to identify the potential causative species.

Following taxonomic revisions of the epiphyte dinoflagellates (*Coolia*) based on the cross-verification of morphological and molecular data, it was considered that the identification results of the species lacking reliable molecular data required further confirmation to prevent confusion in understanding their distribution (Leaw et al., 2016). In this study, both ITS regions and D1-D3 LSU rDNA sequences from *C. palmyrensis* and *C. malayensis* isolates in Korea demonstrated a well-resolved phylogeny with strongly supported statistical values for ML and BI (Figures 4, 5). These phylogenetic trees revealed similar branching patterns and phyletic clades in *Coolia*, which is consistent with previous studies (Jeong et al., 2012;

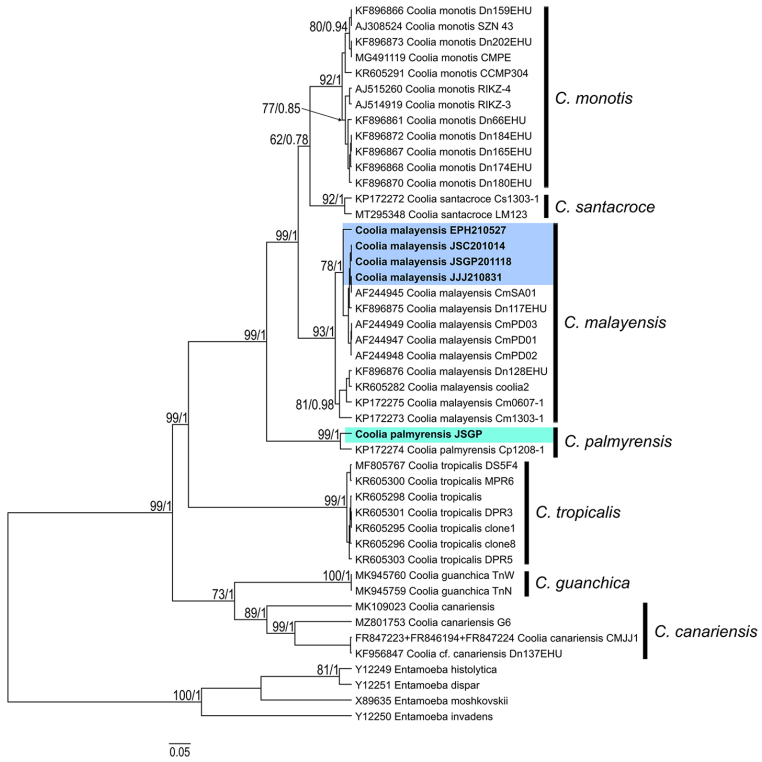

FIGURE 4
Bayesian phylogenetic analysis of *Coolia palmyrensis* and *Coolia malayensis* isolates from Jeju Island and the East Sea of Korea based on the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Numbers at nodes represent bootstrap support values from Maximum Likelihood (ML) on 1000 replicates and posterior probabilities from Bayesian Inferences (BI). The scale bar indicates substitutions per site.

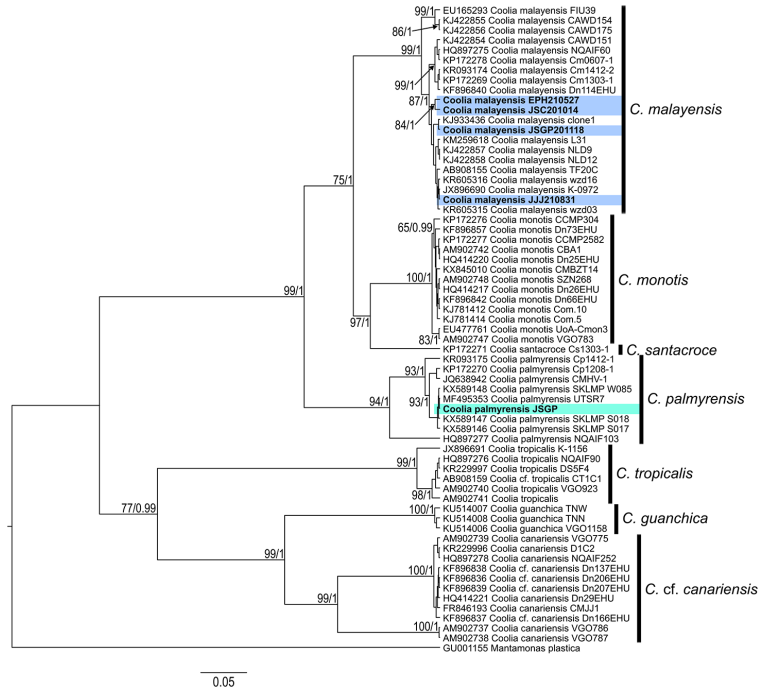

FIGURE 5
Bayesian phylogenetic analysis of *Coolia palmyrensis* and *Coolia malayensis* isolates from Jeju Island and the East Sea of Korea based on the D1-D3 large subunit ribosomal DNA (LSU rDNA) sequences. Numbers at nodes represent bootstrap support values from Maximum Likelihood (ML) on 1000 replicates and posterior probabilities from Bayesian Inferences (BI). The scale bar indicates substitutions per site.

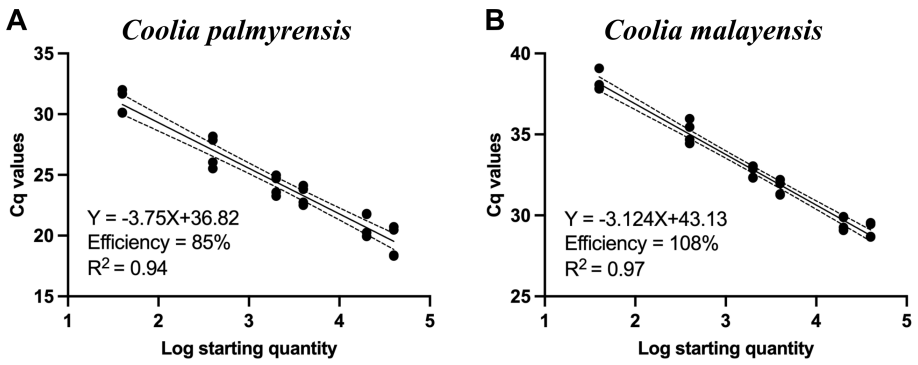

FIGURE 6
Standard curves of **(A)** *Coolia palmyrensis* and **(B)** *Coolia malayensis* for the species-specific quantitative polymerase chain reaction (qPCR) assays with a specific number of cells of genomic DNA (gDNA) extracts from each established clonal culture.

Mohammad-Noor et al., 2013; Karafas et al., 2015; Wakeman et al., 2015; Larsson et al., 2019).

In 2016, Leaw et al. highlighted that identification based on both morphological differences and molecular lineages should be used to establish a more accurate classification of the benthic dinoflagellate taxonomy and the biogeography of *Coolia* species. The species *C. palmyrensis* was observed in various tropical regions: the Great Barrier Reef in Australia in the Southwestern Pacific (Momigliano et al., 2013) and Palmyra Atoll in the Pacific and the Dominican Republic in the Atlantic Ocean (Karafas et al., 2015), indicating that this species is likely restricted to tropical regions within the 30° north and south latitudinal range. Additionally, subsequent studies have confirmed its presence in tropical waters: Long Ke in the eastern waters of Hong Kong at 22° latitude (Leung et al., 2017); Heron Island Lagoon in Australia at 23° latitude (Larsson et al., 2019); Abrolhos Archipelago (Bahia) and Pernambuco in Brazil at 13 and 8° latitude, respectively (Tibiriçá et al., 2020); Pago Bay in Guam (Phua et al., 2021); and Isla San José in the southwest Gulf of California (Morquecho et al., 2022) (Figure 9). These identifications were confirmed based on both morphological and molecular data. In this study, we observed *C. palmyrensis* on Jeju Island, Korea, and identified its morphological and molecular lineages after establishing a clonal culture. This is the first report of *C. palmyrensis* in Korea, with confirmed overwintering populations in 2023. Overwintering cells of the benthic dinoflagellate *Ostreopsis* cf. *ovata* were detected at an average density of 139 cells g^{-1} around Jeju Island, Korea (Park et al., 2020). These observations indicate that these species have adapted to the local environmental conditions in Korea. This adaptation highlights the resilience of these microorganisms and their potential implications in regional ecosystem dynamics. Consequently, *C. palmyrensis* may have integrated into the established benthic phytoplankton communities on Jeju Island. The isolation site in Seogwi is located at 33° latitude in temperate waters.

Because of global warming and rise in seawater temperature, the benthic dinoflagellates are expanding their biogeographic

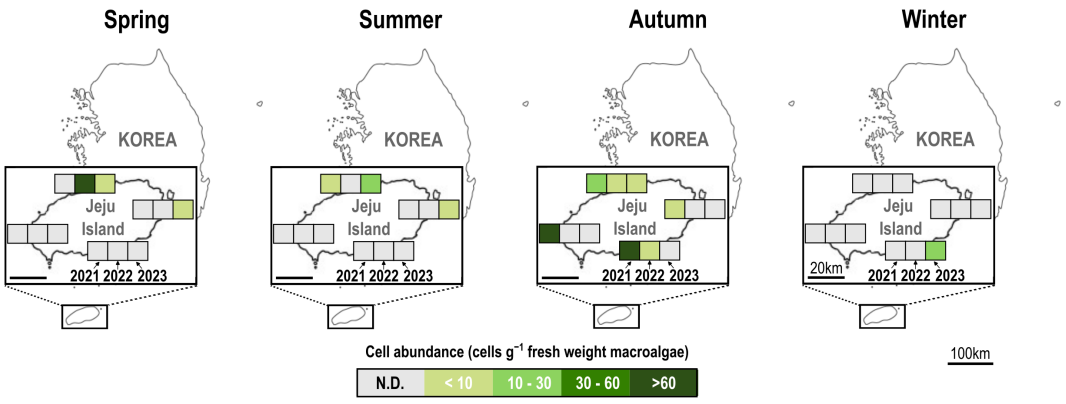

FIGURE 7
Seasonal cell abundance of *Coolia palmyrensis* in macroalgal samples collected from Jeju Island from 2021 to 2023, determined using a species-specific quantitative polymerase chain reaction (qPCR) assay. Samples were collected from four locations on Jeju Island: Dodu (north), Seogwi (south), Seongsan (east), and Sinchang (west). Each square represents the abundance of *C. palmyrensis* cells (cells g^{-1} fresh weight macroalgae) in the respective location and season. The intensity of the color corresponds to the cell density, with darker shades indicating higher cell concentrations. N.D., not detected.

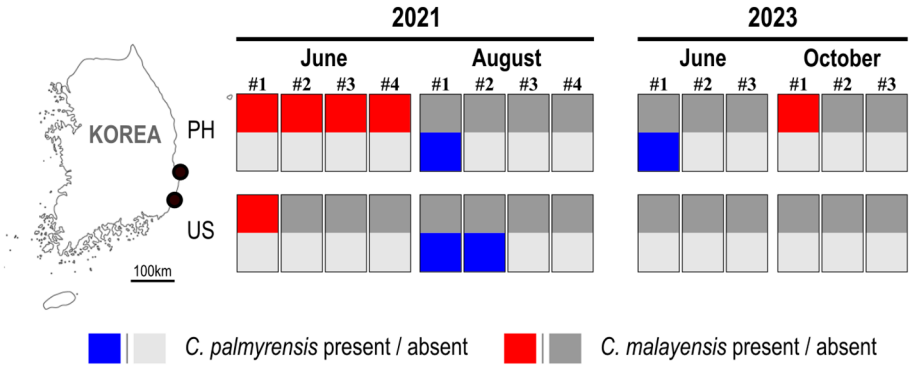

FIGURE 8
Map of the sampling sites in Pohang (PH) and Ulsan (US) showing the occurrence of the benthic dinoflagellates *Coolia palmyrensis* and *Coolia malayensis* in June and August 2021, and June and October 2023. Each numbered label represents individual macroalgal samples collected during that time period. Rectangular boxes correspond to qPCR assay results for these samples: the upper half of each box indicates the presence (red) or absence (gray) of *C. malayensis*, and the lower half indicates the presence (blue) or absence (gray) of *C. palmyrensis*. Light and bold grey colors are used to differentiate the absence of *C. palmyrensis* (light grey) and *C. malayensis* (bold grey).

distribution to temperate waters (Wells et al., 2020). For example, *Gambierdiscus* species produce biotoxins that cause ciguatera fish poisoning (CFP), and were traditionally distributed throughout the tropical and subtropical waters. Recently, these species have been observed in the temperate regions of the Pacific Ocean (Litaker et al., 2010). The species *G. toxicus* was observed on macroalgae with a maximum cell density of 4.7 cells g^{-1} in the brown algae in Ago Bay, Japan, at 34° latitude (Ishikawa and Kurashima, 2010). Similarly, the occurrence of *C. palmyrensis* in the Seogwi area may be a result of habitat expansion of tropical benthic dinoflagellates driven by climate change, facilitating their survival within the thermal tolerance of each species. Our study further supports this hypothesis by revealing a steady rise in seawater temperature along both the eastern coast and Jeju Island in Korea from 2015 to 2021 (Figure 10). During this period, seawater temperatures on the eastern coast of Korea increased by approximately 3.0°C, while in Jeju Island, the increase was around 2.2°C. This consistent warming trend is likely contributing to the northward expansion of tropical benthic dinoflagellates, including *C. palmyrensis*, into temperate regions such as Jeju Island. Warmer temperatures may enhance the survival and reproduction rates of *C. palmyrensis*, allowing it to establish and maintain overwintering populations even in non-tropical waters. Therefore, the habitat expansion of *C. palmyrensis* in Korea appears closely linked to these changing thermal conditions, highlighting the ecological impacts of global warming on marine ecosystems. To fully understand the introduction and

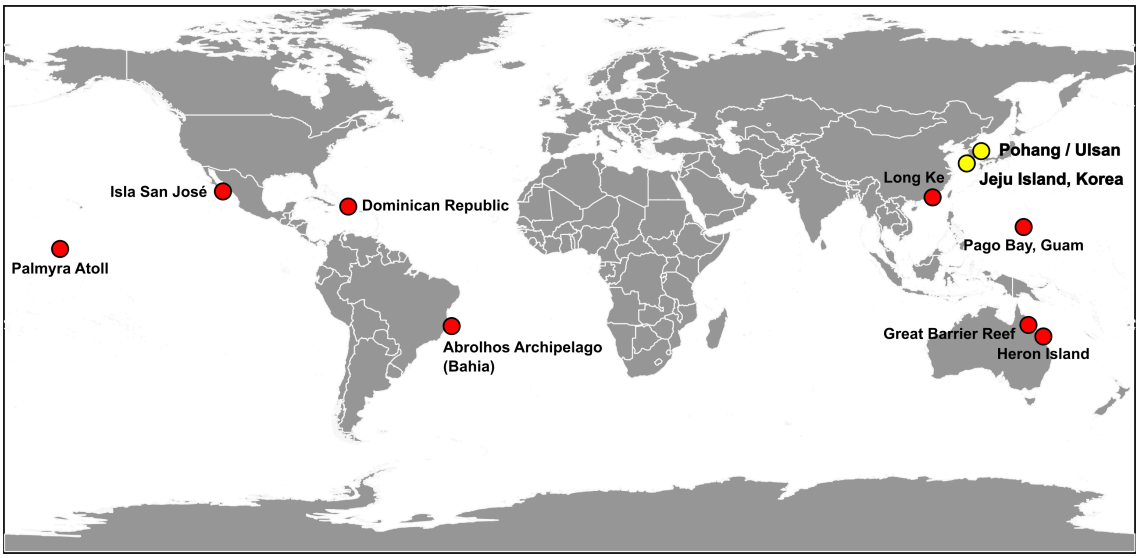

FIGURE 9
Global distribution of *Coolia palmyrensis*. The locations, where each strain has been isolated and identified through a combination of morphological and molecular assessment, are represented with circles. Red circles represent data reported from previous literatures, whereas yellow circles indicate data obtained from this study.

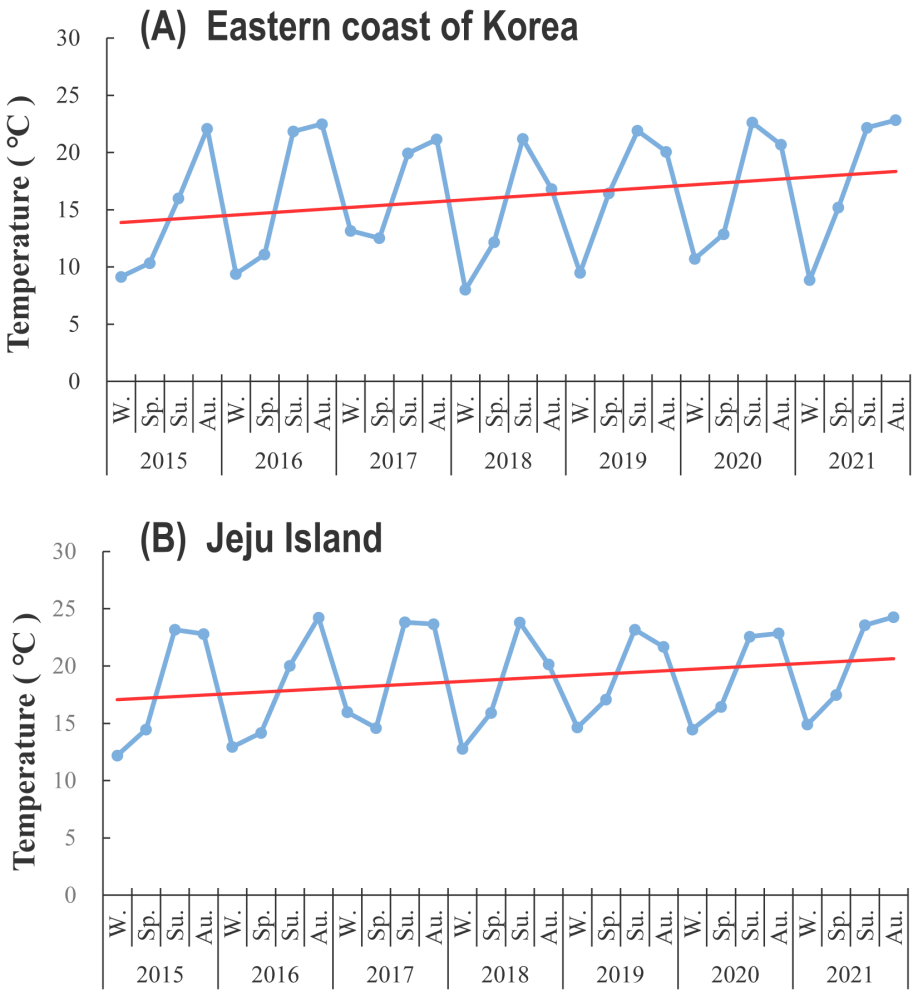

FIGURE 10
Temporal changes in seawater temperature (°C) from 2015 to 2021 at **(A)** the eastern coast of Korea and **(B)** Jeju Island. The blue line represents seasonal temperature variations, and the red line indicates the linear trend of temperature increase over time. The data suggest a total increase in seawater temperature of 3.0°C on the eastern coast and 2.2°C on Jeju Island over the 7-year period. W., Sp., Su., and Au. indicate winter, spring, summer, and autumn, respective.

establishment of *C. palmyrensis* in Korea, further studies are needed to examine the relationship between seawater temperature alterations and their geographic distributions, along with other environmental factors such as the influence of the Tsushima Current and changes in macroalgal species composition. Long-term monitoring considering those factors will be essential to clarify the mechanisms driving the geographic expansion of benthic dinoflagellates in Korean waters.

The presence of epiphytic dinoflagellates in Korean waters was first reported in autumn 2009, with *Coolia* spp. reaching a maximum abundance of 710 cells g^{-1} on macroalgae, which was determined through light microscopy using a Sedgwick-Rafter counting chamber (Kim et al., 2011). In 2012, Korean strains of *C. canariensis* and *C. malayensis* were observed off Jeju Island at 33° latitude, and their identification was confirmed through morphological and molecular analyses (Jeong et al., 2012). However, there has been no further research on the distribution and occurrence of these species in the coastal waters of Jeju Island, or even in Korea.

In this study, although *C. malayensis* cells were not detected in macroalgal samples from Jeju Island using the species-specific quantitative PCR assay during the study period, we were able to isolate and establish a clonal culture of the species from the same macroalgal samples. This suggests that the cell abundance may have been below the detection limit of the qPCR assay, yet sufficient for successful isolation and culturing. Numerous researchers have been interested in understanding the alterations in biogeographic distributions of benthic dinoflagellates in response to ocean climate change (Hallegraeff, 1993, 2010; Aligizaki, 2009; Wells et al., 2020). For example, the species *Ceratium hexacanthum* (currently regarded as *Tripos hexacanthus*) has expanded its geographic range from the south of the British Isles to the North Sea of Scotland (Hays et al., 2005). The widespread distribution of the benthic dinoflagellate *Ostreopsis* from tropical waters to higher latitudes is attributed to its ability to thrive at increased temperatures, driven by global warming (Shears and Ross, 2009; Accoroni et al., 2024). We identified *C. malayensis* in macroalgal samples from the coastal waters of Pohang and Ulsan in the East Sea

of Korea, although the observations were sporadic. These two sites were located further north of Jeju Island at 36 and 35° latitude, respectively. Similarly, *Coolia* was detected in Pohang in 2012, with a maximum cell density of 3 cells g^{-1} using fluorescence microscopy, and this observation was confirmed even in Yangyang, which is further north of Pohang (Baek, 2012). Because benthic dinoflagellates on Jeju Island have migrated from tropical waters to the temperate regions of Korean coastal waters through the Tsushima Current (Lim et al., 2021), the northeastward migration of *Coolia* cells along the Korean Peninsula may be facilitated by the current and its branches, potentially in response to oceanic climate changes. In August 2023, *C. malayensis* cells were observed in plankton net samples collected from Yangyang at a high density of 5367 cells L^{-1}, which was analyzed using qPCR (unpublished data). Additionally, the cells were identified at a similar site in October 2023 at a density of 3 cells g^{-1} in the macroalgae *Amphiroa beauvoisii*. Moreover, at the same time, *Coolia palmyrensis* occurred in Sokcho, which is located further north of Yangyang, at a cell density of 5 cells g^{-1}. The distribution of *C. malayensis* from Jeju Island, where the cells were previously reported by Jeong et al. (2012), to the eastern coastal waters of Korea may be associated with environmental changes driven by climate-related changes. However, the intermittent monitoring results of the two *Coolia* species do not offer conclusive insights. Further studies based on long-term monitoring and measurements of environmental changes, such as the composition of macroalgae in habitats and increasing temperature, are required to clarify the reasons for the changing distributions and understand the migration patterns of *Coolia* species towards the north in Korea.

5 Conclusion

Among the five isolates of *Coolia* species, one was *Coolia palmyrensis* and the other four were *Coolia malayensis*, based on morphological and phylogenetic analyses. This study represents the first report of *C. palmyrensis* in Korea, with qPCR assay monitoring revealing an established population on Jeju Island, specifically during autumn, and overwintering populations in 2023. However, *C. malayensis*, previously observed only on Jeju Island, was not observed at the same sites during the study period. Considering the potential for climate change to shift coastal habitats of benthic organisms, the assessment was expanded to Pohang and Ulsan in the eastern coastal waters of Korea, located further north than Jeju Island. At these sites, a considerable number of *C. malayensis* cells were observed at Pohang sites, alongside the presence of *C. palmyrensis*, as determined through qPCR. Although these observations were sporadic, this study enhances our understanding of the migration patterns of benthic dinoflagellates. Further assessments are required to understand the relationships between the changing distribution of *Coolia* species and other environmental factors in response to climate change.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author. The genetic data produced for this study have been deposited in Genbank, with the accession numbers indicated in Table 3.

Author contributions

J-HH: Writing – original draft, Methodology, Investigation, Data curation. SM: Writing – original draft, Methodology, Investigation. HL: Writing – original draft, Methodology, Formal analysis. JP: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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