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Morphology and genetic analysis of *Coolia malayensis* (Dinophyceae) from the North Viet Nam

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Abstract. In this study, two strains of *Coolia malayensis* isolated from macroalgae *Padina* spp. and *Sargassum* spp. collected in the Gulf of Tonkin, Viet Nam were identified and described using morphological characteristics and phylogenetic analysis. Detailed morphological characterization of *C. malayensis* was described and illustrated with light and scanning electron microscope images. *C. malayensis* from Viet Nam had morphological features similar to previous descriptions of the species, including the 3''' plate being larger than the 4''' plate. However, the shape of the 3' plate of *C. malayensis* from Viet Nam is pentagonal, while it is quadrangular in the original description from Malaysia. In phylogenetic analyses of the LSU rDNA (D8-D10 region), the Vietnamese strains of *C. malayensis* were grouped with the Macauley Island (New Zealand) strain and the strain of Coolia sp. from Japan with full support value (ML = 100 and BI = 1.0). The intraspecific pairwise distance (p-distance) and the number of different nucleotides among all *C. malayensis* strains from Viet Nam, New Zealand and Japan ranged from 0 % (0 bp) to 0.12 % (1 bp), and from 0.25 % (2 bp) to 0.37 % (3 bp), respectively.

Keywords: *Coolia malayensis*, Gulf of Tonkin, LSU, morphology, Viet Nam.

Classification numbers: 3.1.2, 3.6.1.

1. INTRODUCTION

The *Coolia* species are marine, benthic dinoflagellate with world-wide distribution from temperate to tropical waters, they are associated with macroalgae, seagrasses, sandy bottoms [1–3]. The dinoflagellate genus *Coolia* was established by Meunier [4] with *C. monotis* Meunier as the type and only species. At present, eight *Coolia* species have been identified and described, namely *C. monotis*, *C. tropicalis, C. areolate, C. canariensis, C. malayensis, C. santacroce, C. palmyrensis*, and *C. guanchica* [5]. Cells of these *Coolia* species are highly alike in shape, size, numbers and arrangement of epithecal and hypothecal plates. Among them, the *C. malayensis* is morphologically very similar to *C. monotis.* The key morphological features used to distinguish species in the genus *Coolia* are based on the shape, size, thecal plate arrangement, and ornamentation [6–9].

The species diagnosis within genus *Coolia* was traditionally based on the epithecal tabulation. The recently reported species, the *C. palmyrensis*, *C. tropicalis, C. areolata, C. canariensis,* and *C. guanchica* share the feature that the 1' plate is located at the centre and the largest in the epitheca. Morphology was examined by light microscopy (LM), scanning electron microscopy (SEM), and sometimes by transmission electron microscopy (TEM). Phylogenetic analysis based on sequences of ITS (ITS1, 5.8S, ITS2 regions) and/or LSU (D1-D2 or D1-D3 regions) [1–3, 8, 10–16].

In Viet Nam, there are few studies on the taxonomy of the genus *Coolia*, three species of *Coolia monotis, C. tropicalis,* and *C. canariensis* were identified and described using only light and scanning electron micrographs [16–19]. Recently, the *C. malayensis* was reported in Nha Trang Bay, South Central coast of Viet Nam [20]. As mentioned above, the morphology of species *C. monotis* is very similar to that of species *C. malayensis,* therefore, their classification would be very difficult without genetic analysis support. In this study, the *C.malayensis* was described based on morphology and genetic characteristics of two strains isolated (VNTM005 and VNHG008) in the Gulf of Tonkin.

2. MATERIALS AND METHODS

2.1. Sampling locations

The survey was carried out in May 2021 aboard the research vessel "Akademik Oparin" in the Gulf of Tonkin, including Thanh Mai Island (21.03810°N – 107.82365°E) in Quảng Ninh province (Viet Nam), on 14 May 2021 and Hon Gio Island (17.91201°N – 106.67337°E) in Quang Binh province (Viet Nam), on 25 May 2021 (Figure 1).

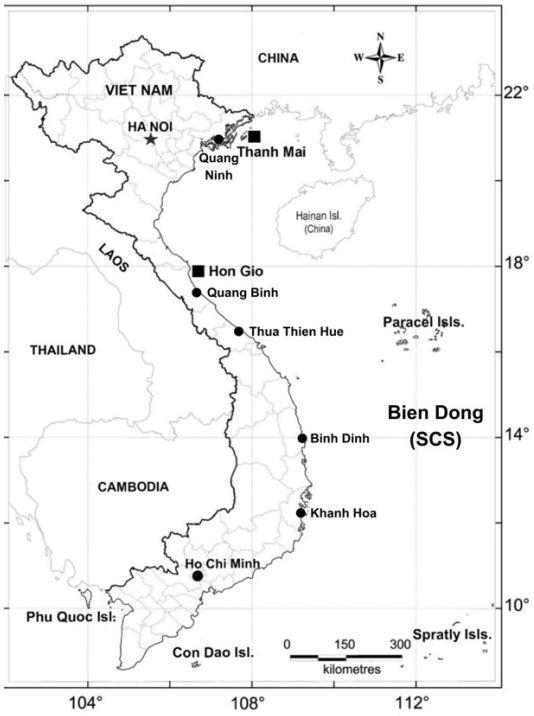

Figure 1. Map of Viet Nam showing two sampling sites (solid squares).

2.2. Sampling, isolation, and culturing of *Coolia malayensis*

For the isolation and culture of *C. malayensis*, the marine macro-algae including *Padina* spp. and *Sargassum* spp. were collected by SCUBA divers at depths of 2-5 m. The macroalgal samples were placed in plastic bags, stored in cool boxes. These bags were mixed and lightly shaken with filtered seawater taken from the same site to dislodge the epiphytic microalgae. The materials were filtered through a series of consecutive meshes with sieve sizes of 125 µm, 64 µm, 32 µm, and 20 µm to remove large particles. Materials on 64 µm, 32 µm, and 20 µm sieves were examined and observed using a Leica MZ 12 stereo microscope. Cells of *Coolia* were sought and isolated by a pipette and maintained in 50 mL flasks containing T-medium. In the laboratory, cultures were maintained in 50 mL flasks containing T-medium placed in an Environmental Chamber (MLR 351, Sanyo, Japan) under a salinity of 32 psu, temperature of 26 °C, light intensity of 35 µmol photons·m^{-2}·s^{-1}, and light : dark cycle of 12 : 12. The wild and cultured cells of *Coolia* spp. were observed alive or fixed by light microscopy. Clonal isolates were established from two of the four sampling sites and confirmed with morphological and molecular analyses to be *C. malayensis* (strains VNTM005 and VNHG008).

2.3. Morphological analysis of *Coolia malayensis*

Light microscopy (LM) of samples was carried out using an epifluorescence microscope equipped with phase contrast and differential interference contrast optics (Olympus BX53F, Tokyo, Japan). The tabulation of the cells was analyzed using Calcofluor White M2R [21]. A digital camera, DP74 (Olympus, Japan), was used for micro-photography.

For SEM examination, cells of *Coolia malayensis* were isolated by Pasteur pipette from preserved samples under a stereomicroscope and placed on a 5 µm carbon membrane filter, rinsed with distilled water, dehydrated through an ethanol series of 15 %, 30 %, 50 %, 70 %, 2 x 96 %, and 2 x absolute ethanol and air-dried. The filter was mounted onto an aluminum stub with carbon tape and finally coated with gold. The stubs were examined on a Zeiss (Jena, Germany) at the Biotechnology Center of Ho Chi Minh City, Viet Nam.

The size of the cell and thecal plates were measured using LM and SEM micrographs. Morphometric features, including the length (L) of the cell, were measured from the apex of the epitheca to the antapex of the hypotheca; the width (W) of the cell was measured directly in front of the cingulum or behind it, and the depth (D) was measured in its widest part in lateral view. Thecal plate terminology follows the Kofoid tabulation system [22], modified by Balech [23] to produce the currently employed plate convention.

2.4. Molecular analysis of *Coolia malayensis*

2.4.1. DNA extraction, PCR amplification, and sequencing

About eight to ten clonal cultures of putative *Coolia malayensis* cells were collected by micropipettes using a dissecting microscope (Leica, MZ12, Solms, Germany). They were then transferred to a 1.5-mL tube containing 10 µL of distilled water. The tube was stored at -72 °C for 10–30 minutes after adding 30 µL of 10 % Chelex 100 (Sigma-Aldrich, St. Louis, USA) buffer to extract the DNA. The samples were then stored frozen. DNA extraction for cultures was performed by exposing the samples to high temperatures (94 °C) for 10 minutes, and afterward, the samples were kept at 4 °C.

Samples containing DNA were used as templates for PCR reactions. The D8-D10 region of 28S ribosomal RNA was amplified using primer pair (forward FD8 and reverse RB) as previously described [24]. In addition, the internal primers GLD8_421F and GLD8_677R were used for sequencing [25]. The total PCR volume of 25 µL included 12.5 µL of 2 x OneTag® Master Mix (New England Biolabs, Ipswich, MA, USA), 10-30 ng of template DNA, and 1 pmol of each primer. The PCR reaction was performed in an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster, CA, USA). The following protocol was used on a thermocycler: Starting with a denaturation step at 96 ℃ for 5 minutes to induce DNA denaturation, followed by 35 cycles of denaturation at 96 ℃ for 30 seconds, annealing at 56 ℃ for 45 seconds, and extending at 68 ℃ for the 60 seconds. After 35 cycles, the sample was incubated at 68 ℃ for 5 minutes before the end of the reaction.

The PCR products were checked by 1 % agarose electrophoresis with EtBr (Merk, Germany). The size of the clear bands was estimated using the standard 1 Kb plus DNA Ladder scale (Thermal Fisher Scientific, MA, USA). The PCR products were purified using the GenEluteTM PCR Clean-Up kit (Sigma-Aldrich, St. Louis, MI, USA) according to the manufacturer's instructions before sending them for sequencing at 1st BASE (Selangor, Malaysia). Sequencing was carried out in both directions (5'-3' and 3'-5'). Each sample was repeated twice, including PCR reaction and sequencing. Clone Manager 9 software (Sci-Ed, Cary, NC, USA) was used to check the quality and standardize the final sequence.

2.4.2. Phylogenetic analyses

Sequences of *Coolia malayensis* were retrieved from GenBank (www.ncbi.nlm.nih.gov) and were compared to existing sequences as described previously [26]. The D8–D10 LSU rDNA data set including two newly generated sequences obtained in this study (GenBank accession numbers OQ932985 and OQ932986) and 39 other sequences of *Coolia* (02 sequences), *Gambierdiscus* (25), *Ostreopsis* (11). *Prorocentrum micans* (AY822609) was selected as the outgroup (Supplementary Table S1). A CLUSTAL W method aligned the dataset using MEGA v. 11 software [27]. jModeTest version 2.1.6 [28] with Akaike Information Criterion (AIC) correction method was used to find the most suitable evolutionary model for the data, increasing the accuracy of the phylogenetic tree. This study uses two evolutionary tree-building methods: Maximum Likelihood (ML) and Bayesian Inference (BI). ML was performed using RAxML version 8.1 [29] with 1,000 bootstrap replications and BI (Metropolis-coupled Markov chain Monte Carlo method) in MrBayes v.3.2.2 [30]. Differences in the number of nucleotides and the evolutionary distance (p-distance) were calculated using MEGA v.11 software [27].

3. RESULTS AND DISCUSSIONS

3.1. Morphological observation

Coolia malayensis Leaw, P. -T. Lim & Usup 2001.

Description: Cell shape is almost spherical in ventral view (Figure 2G) and obliquely in lateral view (Figure 2J). Cell sizes are 23 to 27.5 µm in length (24.7 ± 1.5 µm, n = 8), 23 to 27.8 µm in width (25.4 ± 1.5 µm, n = 11), and 22.1 to 31.1 µm in depth (27.3 ± 2.56 µm, n = 12) (Table 1). The epitheca's first apical plate (1') is narrow, oblong, and hexagonal (Figures 2B-D, G-I). The second apical plate (2') is narrow (Figure 2E), elongated, the smallest in the apical series, and is contiguous to the Po along its left and dorsal sides (Figure 2I). The apical pore

complex (APC) slightly curved, with a length varying from 5.7 to 8.1 μm (Figures 2E & I). The third apical plate (3') is pentagonal (Figures 2D, H, and I), contacting the plates 1', Po, 2', 4", 5", and 6" (Figure 2I). The first precingular (1") plate is small rectangular, and touch with plates 1', 2", and 7" (Figures 2C, D, and G-I). The sixth precingular 6" plate is the largest pentagonal, occupying nearly half of the epitheca (Figures 2B-D, G-I). The seventh precingular plate (7") is pentagonal (Figures 2D, G-H) with a width-to-length ratio of 1.1 to 1.8 μm (1.4 ± 0.3 μm, n = 6) (Figures 2D, G-I, Table 2). In the hypotheca, the third postcingular plate 3"' is the largest, quadrangular, larger than the 4"' plate (Figures 2K-L). Plates 2"' and 5"' are smaller, and plate 1"' is the smallest in the postcingular series (Figures 2F & L). The first and second antapical plates (1"" and 2"") are small and in contact with the posterior part of the sulcus (Figure 2F). The surface of the thecal plates is smooth and irregularly scattered with round pores (Figures 2G-L).

Type locality: Kota Kinabalu, Sabah, Malaysia.

Habitat: Coolia malayensis was found on brown seaweeds, Sargassum and Padina.

Distribution: In Vietnamese waters, *C. malayensis* is recorded in waters of the Gulf of Tokin, the northern marine regions of Viet Nam (for this study), and Nha Trang Bay [17]. Previously, *C. malayensis* was found in Malaysia [8], Korea [9], the Caribbean Sea, Florida, and the Caribbean Islands [31], Thailand [32], Japan [33], Palmyra Atoll [10], Hong Kong [13], New Zealand [26], New South Wales [34], China [3], Brazil [2, 16], the Mediterranean Sea [15], the Gulf of California [11], and Cook Islands [14].

Strains: VNTM005, VNHG008.

Remarks: Morphology of *Coolia malayensis* is very similar to *C. monotis* Meunier, particularly on the epithecal tabulation. However, in *C. malayensis,* the 3"' plate is larger than the 4"' plate in size, whereas in *C. monotis*, the 3"' plate and the 4"' plate are almost equal in size. Jeong *et al.* [9] found cells with the 3"' plate being the same as the 4"' plate.

Coolia malayensis is the most widely distributed species, found in tropical and temperate waters [9, 13, 15]. Until now, the *C. malayensis* has been reported in many Pacific and Atlantic Oceans coastal areas since it was first found in Malaysia [8]. Several studies indicated that *C. malayensis* also found in Korea [9], Thailand [32], Japan [33], Hong Kong [13], Palmyra Atoll [10], New Zealand [26, 35], New South Wales [34], China [3], the Gulf of California [11], Cook Islands [14], Brazil [2], Caribbean Sea, Florida and the Caribbean Islands [31]; and in the Mediterranean Sea [15]. The *Coolia malayensis* is also recorded in Nha Trang Bay [17] and the Gulf of Tonkin, Viet Nam.

Based on morphological observations, Hoppenrath *et al.* [36] suggested that the *C. malayensis* is a synonym of *C. monotis.* However, the separation between these two species is now more widely accepted, largerly based on phylogenetic data [10, 37]. The *Coolia malayensis* and *C. monotis* were separated based on the relative size between the 3"' and 4"' plates on the hypothecal and the ratio of width/ length of the 7" plate on the epithecal [8, 9]. Based on the cell size, apical pore size, and the size and density of pores, Karafas *et al.* [10] also indicated that the *C. malayensis* and *C. monotis* are distinct species. Some strains previously designated as *C. monotis* have been reassigned to the *C. malayensis* [37]. The *Coolia malayensis* was quite distinct in both morphology and genetics from other species (not applicable for *C. areolate*) within the genus *Coolia* (e.g., [8, 10, 12, 37]).

In this study, cell size and morphologies of the Vietnamese other strains of *C. malayensis* in previously studies are summarized in Table 1. The overall shape of the *C. malayensis* Vietnamese strains fit well with the original description [8], except for the shape of the 3' plate. The ranges of the length and width of cells of Vietnamese strains of the *C. malayensis* (23 to 27.5 µm and 23 to 27.8 µm, respectively) were smaller than those of the Malaysian (28 to 33 µm and 27 to 32 µm, respectively) and Brazilian strains (26.7 to 38.8 µm and 25.6 to 37.5 µm, respectively). The strains of *C. malayensis* from Viet Nam had plate 3' in pentagonal shape with its wide contact with plate 5". These characteristics from Vietnamese strains are similar to Korean, Thailand, New Zealand, Japanese, Brazilian, and Mexican strains of *C. malayensis* [9, 11, 12, 26, 32, 33]. In original description, the 3' plate was described as quadrangular without contacting plate 5" [10]. This feature was not clear in the original SEM photographs, but only in the drawing. Besides, the 3' plate was reported to be quadrangular [2, 8, 15, 37], and pentagonal or hexagonal [10] (Table 1).

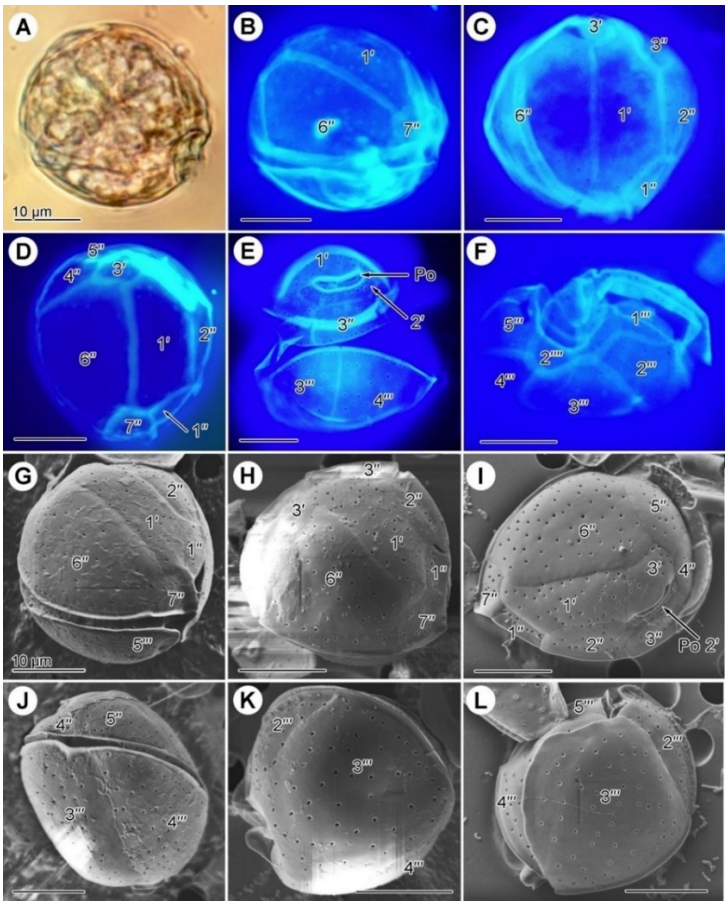

Figure 2. LM and SEM micrographs of *Coolia malayensis.* A-F Light microscopy. A (PC) - B (Epi.). The right lateral view shows the spherical outline of a cell (A) and the epithecal plates (B). C-D (Epi.). Cells in the apical view showed the epithecal plates. E (Epi.). Theca in the left lateral view showed the long 2' plate and the apical pore complex (APC) with a narrow apical pore. F (Epi.). The hypotheca in the ventral view showed the sulcal plates and hypothecal plates. G-L. Scanning electron microscopy. Showing the surface of the thecal plates is smooth and irregularly scattered with round pores. G. Cell right lateral view showing the epithecal plates and cingulum. H-I. Cells in the apical view showed the epithecal plates. J. Cell in right lateral view showing the epithecal and hypothecal plates. K-L. The hypotheca showing plates with the 3"' plate being the widest in hypothetical plates. All scales = 10 µm.

Table 1. Comparison of the morphometric features of *Coolia malayensis* strains isolated from the Gulf of Tonkin (Viet Nam) with isolates from other areas.

Sampling locality	Length (L) (µm)	Width (W) (µm)	7" W: L	Po length (µm)	Shape 3' plate	Relative size of 3"' to 4"' plates	Reference
Gufl of Tonkin, Viet Nam	23-27.5	23.1-27.8	1.1-1.8	5.7-8.1	Pentagonal	3"'> 4"	This study
Port Dickson, Kota Kinabalu, Langkawi Island Malaysia	28–33	27–32	1.2–1.5	5	Quadrangular	3"'> 4"'	[10]
Jeju Island, Korea	25.4-36.8	24.0-35.4	0.8-2.0 0.9-2.1	3.4-7.4 4.3-6.8	Pentagonal	3"'> 4"	[8]
Offshore NC, St. Croix, Dominican Republic, Pacific Ocean	19.3–28.8	22.7–31.5	1.3–1.6	5.3–6.8	Pentagonal; Hexagonal; Wedgendshaped	3"'> 4"	[38]
Pacific and Atlantic Oceans	19–31	19–32	1.22–1.65	4.7–9.3	Quadrangular	3"'> 4"	[9]
Andaman Sea, Thailand	19.6–33.6	19.4–33	0.84–1.75	4–7.4	Pentagonal	3"'> 4"	[30]
Northland, New Zealand	nd	nd	nd	nd	Pentagonal	3"'> 4"	[25]
Okinawa, Japan	20–32	22–33	1.4	nd	Pentagonal	3"'> 4"	[32]
Brazil and Puerto Rico	22–33	19–33	nd	6–9	Quadrangular	3"' = 4"	[2]
Bahia, Brazil	26.7–38.8	25.6–37.5	nd	6	Pentagonal	3"'> 4"	[36]
Brazil	20.0–27.5	22.8–26.5	1–2.2	3.0–7.1	Pentagonal	NA	[11]
Brazil	16.6–25.3	19.6–29.3	nd	6.3 ± 0.5	NA	NA	[15]
Hainan Island, China	20.9–34.3	18.0–32.3	1–1.4	5.3–7.4	NA	NA	[3]
Gulf of Gabès, Tunisia	22–26	25–30.9	1.2–1.5	4–7.3	Quadrangular	3"'> 4"	[14]
Bahía de La Paz, Mexico	23-44	23-39	1-2	5-11	Pentagonal	3"'> 4"	[31]

* NA: Not available; nd: not identify.

Morphological observations on our isolation from Thanh Mai and Hon Gio Islands in the Gulf of Tokin, Viet Nam, show that the 3”’ plate is larger than the 4”’ plate, which is well matched with that described for *C. malayensis* strains from previous studies [8, 15]. The ratio of width to length of the 7” plate in the Vietnamese strains was 1.1-1.8. In both LM and SEM micrographs, the ranges of the Po length (5.7-8.1 μm) were the same as previously reported strains. These results conclude that these isolations are morphologically similar to *C. malayensis* previously reported in other regions (Table 1).

3.2. Phylogenetic analysis

Based on the primers used, the size of the D8-D10 LSU rDNA fragment was around 1.100 bp. Previous studies have used lengths of 810 – 890 pb to analyze the phylogeny of *Coolia* and the relative genera. Therefore, this study used an 818 bp dataset for analysis. The final LSU rDNA D8-D10 alignment contained 41 sequences of 818 bp long, of which 476 bp (58.2 %) were conserved sites, 338 (41.3 %) were variable sites, 262 (32 %) were parsimony informative sites, and 76 (9.3 %) were singletons. The results of the Bayesian Inference (BI) and Maximum Likelihood (ML) analyses based on the LSU rDNA D8-D10 sequences showed three distinct clades for each genus (*Gambierdiscus*, *Coolia*, and *Ostreopsis*). These clades were distinguished and fully supported, 100 % (bootstrap value), and 1.0 (posterior probability), respectively. The results show that the two strains of *C. malayensis* collected from Viet Nam (VNTM005, VNHG008) and the known the *C. malayensis* in Macauley Island, New Zealand (MF109031), *Coolia* sp. (AB674901) from Japan formed a distinct clade with very high support values (100 % and 1.0, Figure 3). The intraspecific pairwise distance (p-distance) and the number of different nucleotides among all collections of *C. malayensis* from the New Zealand, Japan, and Viet Nam based on LSU (D8-D10) sequence analyses ranged from 0 % (0 bp) to 0.37 % (3 bp) (Table 2). There were no nucleotide differences between the two strains collected in Viet Nam (Table 2).

Table 2. Estimates of evolutionary divergences (p-distances, ranges) and number of different nucleotides (shaded cells) among strains of *Coolia malayensis* based on 818 bp of the D8–D10 region of the LSU rDNA gene.

	Coolia malayensis			*Coolia* sp.
GenBank	OQ932985	OQ932986	MF10901	AB674901
OQ932985		0.12	0.12	0.37
OQ932986	1		0	0.25
MF10901	1	0		0.25
AB674901	3	2	2	

There may be limitations to differentiating species in the genus *Coolia* based on morphological characteristics. Therefore, with the molecular sequencing technology and accompanying phylogenetic analyses, it is becoming increasingly apparent that ostensibly cosmopolitan species often encompass a complex of cryptic species [10]. The strains of *C. malayensis* were distinguished from other species in the genus *Coolia* based on morphological analysis combined with molecular phylogenetics reported by other authors [37, 39]. Phylogenetic analysis based on sequences of ITS (ITS1, 5.8S, ITS2) or LSU (D1-D2 or D1-D3 regions) or both were performed in several studies [8, 10, 11, 14, 16]. The phylogenetic tree

from this study was constructed based on LSU rDNA (D8-D10 region) sequences supported by Rhodes *et al.* [35].

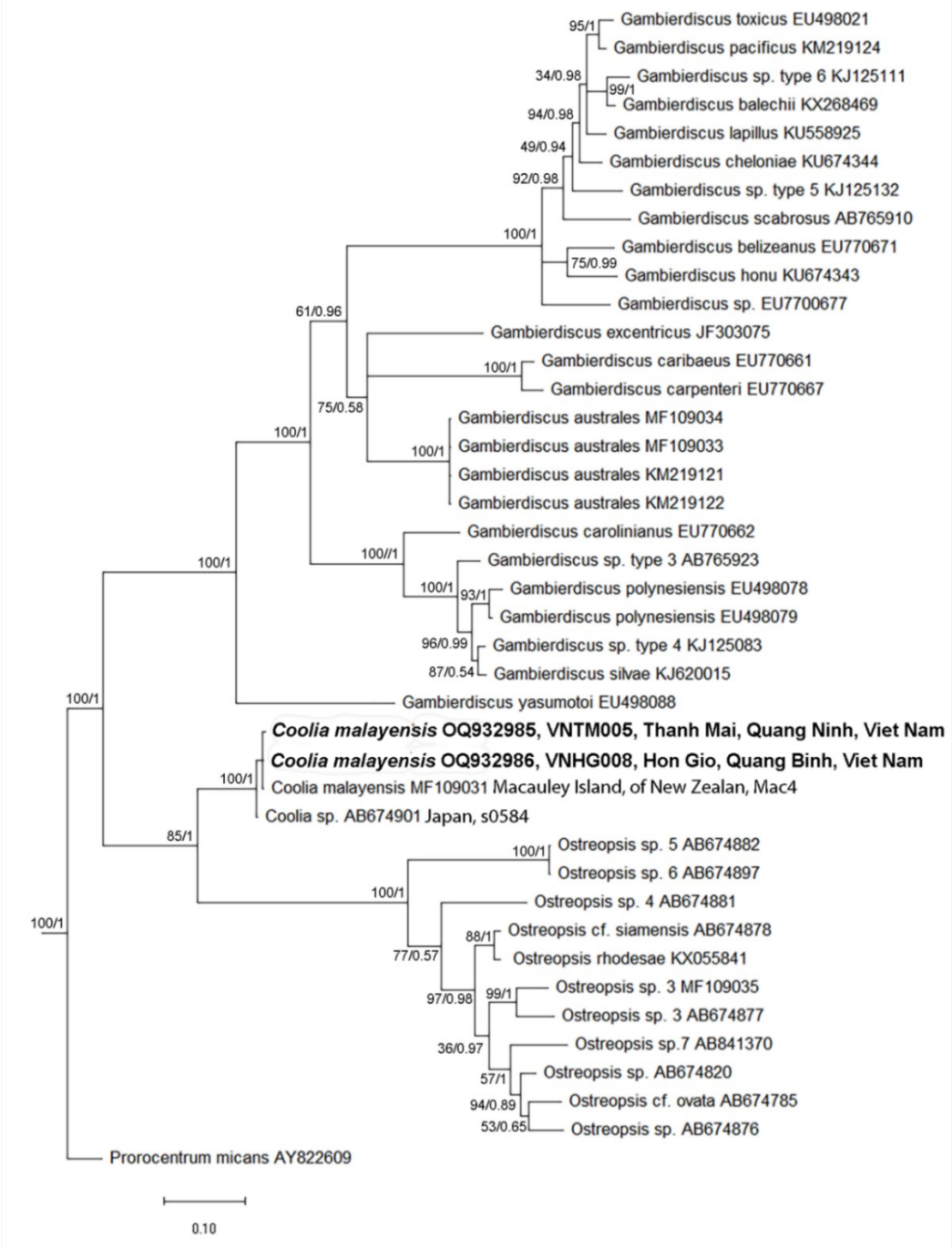

Figure 3. Phylogenetic analysis of large sub-unit (LSU, D8–10 region) sequences obtained from *Coolia malayensis* (in bold) isolated during this study (Species name, GenBank accession number, strain code, and place of origin). *Prorocentrum micans* was used as an outgroup. Bootstrap support values from Maximum Likelihood (ML) and posterior probabilities from Bootstrap Inferences (BI) values are shown at each node: ML (left) and BI (right). The scale bar indicates substitutions per site.

The trains of *C. malayensis* isolate from the North of Viet Nam were geographically located about 9,000 km from Macauley Island of New Zealand. However, there were no nucleotide differences in LSU rDNA (D8-D10 region) sequences between the Vietnamese strains and the strain collected from Macauley Island of New Zealand. Thus, a great molecular diversity of *C. malayensis* may still be unveiled in the Pacific Ocean, and this long-range raises new questions about biogeography, ecology, physiology and evolution of the *C. malayensis* and other areas.

4. CONCLUSIONS

Similar to other epiphytic dinoflagellate genera, species within this genus can be very similar in morphology and may only be identified using supporting molecular markers. In this context, the *Coolia monoti*s and *Coolia malayensis* are two species that pose such challenges. The present study provides both detailed information on the morphological features of species *Coolia malayensis* in light and electronic scanning microscopy and genetic data of the LSU rDNA D8-D10 region, contributing to the regional biodiversity data. Besides, this is to confirm the morphological features of the pentagonal shape of the third apical plate (3') of Vietnamese strains of *Coolia malayensis*.

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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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