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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目： Studies on toxic benthic dinoflagellates from Thailand
Title of Dissertation (タイ産の底生性有毒渦鞭毛藻に関する研究)

学位論文要約：
Dissertation Summary

Benthic dinoflagellates constitute a group of special interest since they comprise a high number of toxic and/ or potentially toxic species. Some species of the genera *Ostreopsis, Gambierdiscus* and *Coolia* can produce toxins that are thought to be associated with human health problems. For instance, the species of *Ostreopsis*, which can produce palytoxin and its analogs, are known as causative agents of respiratory illness and skin irritations in Mediterranean Sea (Tichadou et al., 2010) and the species of *Gambierdiscus* are causative agents for ciguatera fish poisoning (CFP) which affects between 50,000 and 500,000 people per year worldwide (Fleming et al., 1998). In tropical areas, the dinoflaglellates genera *Ostreopsis* and *Coolia*, which usually form mixed assemblages with *Gambierdiscus*, have been thought to be causative organisms of CFP by some researchers (Yasumoto et al., 1987, Holmes et al., 1995; Faust, 2009).

CFP is the main cause of non-bacterial illness associated with seafood consumption worldwide (Baden et al., 1995; Dickey and Plakas, 2010; Tester et al., 2013). Research has shown that the lipophilic ciguatoxins produced by *Gambierdiscus* species bioaccumulate via marine food web and causes disruption of gastrointestinal, cardiovascular and neurological functions, with symptoms ranging from mild to severe and occasionally resulting in death (Friendman et al., 2008). In 1984, there was a case of CFP of an Italian visitor who had ingested ocean fish in Thailand (Sozzi et al., 1988). Saraya et al. (2014) reported the six patients in Thailand (2 patients in Bangkok in 2007 and 4 patients in Phuket in 2009) showed symptoms of ciguatera after consumption the unidentified marine fish portion. Considering these issues, it is very important to conduct studies on the causative dinoflagellates, *Ostreopsis*, *Gambierdiscus* and *Coolia*, which have been considered as causative organisms of CFP.

Currently, harmful algal blooms can spread with ocean currents, so one country's problem can easily spread to its neighbors in some case (Holmes and Teo, 2002). Previous studies showed that *Ostreopsis, Gambierdiscus* and *Coolia* strains were isolated from Malaysian coastal waters (Holmes et al., 1998; Leaw et al., 2001; Mohammad-Noor et al., 2006). Limited records indicate that the three genera *Ostreopsis*, *Gambierdiscus* and *Coolia* are present in Thai coastal waters. For example, an occurrence of *Ostreopsis* spp. was reported in a mangrove forest and shrimp pond in the upper Gulf of Thailand , *G. toxicus* and an unidentified *Gambierdiscus* sp. were reported in the shrimp pond in the upper Gulf of Thailand, and *C. tropicalis* was found at Surin Island in the Andaman Sea (Piumsomboon et al., 2001). To date, none of the reports, however, has undertaken the detailed morphological and molecular studies to identify them from Thailand. To obtain useful information for

understanding potential problem by benthic dinoflagellates in Thailand, studies on the species composition and distribution were conducted.

In this study, we established the strains of *Ostreopsis*, *Gambierdiscus* and *Coolia* from samples collected along Thai coasts (Fig. 1). Their morphologies were observed using light microscope (LM) and/or scanning electron microscope (SEM) in order to obtain knowledge about their taxonomy for morphological identification. To reveal phylogenetic relationship and at the same time support the morphological identification, the LSU rDNA D8/D10, D1/D2 or ITS-5.8S rDNA sequences of *Ostreopsis*, *Gambierdiscus* and *Coolia* were used for the phylogenetic analyses. Furthermore, we clarified the distribution of them in Thailand based on the phylogenetic data of the each species and the location from which they were isolated.
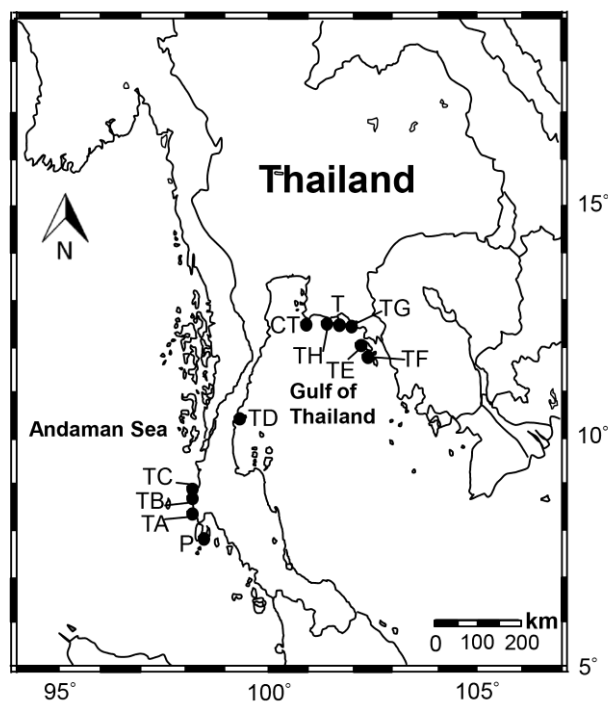

Figure 1 Map of Thailand showing sampling sites in the Gulf of Thailand and the Andaman Sea. Each location is indicated by the following code: CT, Koh Raed, Chonburi; P, Ao Tang Khen, Phuket ; T, Koh Man, Rayong; TA, Hat Thai Mueang, Phang-Nga; TB, Khao Lak, Phang-Nga; TC, Leam Pagarung, Phang Nga; TD, Hat Sai Ri, Chumphon; TF, Koh Wai, Trat; TE, Koh Chang, Trat; TG, Khao Leam Ya, Rayong; TH, Ao Khung Wiman, Chanthaburi.

The genus *Ostreopsis* from Thailand

Based on LM observation, all Thai *Ostreopsis* cells had numerous golden colored plastids and live cells swim with a geotropic orientation. In the apical view, a characteristic oblong and tear-shaped form was observed in the cells of all the Thai *Ostreopsis* strains examined. The representative strains of each clade/subclade to which *Ostreopsis* spp. from Thailand belong were examined for the morphological analysis. Thai *Ostreopsis* cells displayed a similar plate pattern of Po, 3′, 7″, 5‴, 1p and 2⁗, which is common to all *Ostreopsis* species. (data not shown). In this study, morphometric information of Thai *Ostreopsis* strain were measured separately following the results of phylogenetic analyses, showing four *Ostreopsis* species/clades described. Morphological features of the two subclades of *O.* cf. *ovata* (Thailand and South China Sea subclades) such as cell shape, smooth thecal surface and thecal plate pattern were similar in that of *O. ovata* described by Fukuyo (1981). Most cells of *Ostreopsis* sp. 6 differed from those of the two subclades of *O.* cf. *ovata* and *Ostreopsis* sp. 7 cells in having an undulated shape in the lateral view. The results of morphometric information revealed that the DV and DV/W ratio in the two subclades of *O.* cf. *ovata* from Thailand were smaller than that of the *O. ovata* cells (Fukuyo, 1981). Dorso-ventral diameter (DV) and trans-diameter (W) of the four strains (T153, T194, T197 and TD7OS)

of *O.* cf. *ovata* Thailand subclade ranged from 20.3 to 47.8 μm (average ± standard deviation: 35.1 ± 3.2 μm) and from 15.2 to 39.8 μm (average ± SD: 24.5 ± 2.0 μm), respectively. The cells of five strains (TF5OS, TF20OS, TF21OS, TF24OS and TF32OS) of *O.* cf. *ovata* South China Sea subclade had DV ranging from 24.1 to 56.8 μm (average ± SD: 38.8 ± 3.8 μm) and W ranging from 15.8 to 47.4 μm (average ± SD: 27.5 ± 3.3 μm). Five strains (TB17OS, TB20OS, TB30OS, TB32OS and TB37OS) of *Ostreopsis* sp. 7 had DV ranging from 21.5 to 51.6 μm (average ± SD: 36.0 ± 2.6 μm) and W ranging from 14.7 to 40.1 μm (average ± SD: 25.2 ± 2.2 μm. Furthermore, the sizes of cells of *Ostreopsis* sp. 6 strain TF29OS isolated from Thailand were the largest in this study. The sizes of DV and W values were 49.9 to 84.3 μm (average ± SD: 62.4 ± 8.0 μm) and 35.4 to 66.9 μm (average ± SD: 48.2 ± 5.5 μm), respectively. Based on culture observations, average DV/W ratio of *Ostreopsis* sp. 6 was 1.30 ± 0.05. On the other hand, those of *O.* cf. *ovata* Thailand subclade, South China Sea subclade and *Ostreopsis* sp. 7 were 1.43 ± 0.05, 1.42 ± 0.05 and 1.43 ± 0.09, respectively. No statistical significance differences of DV, W and DV/W ratio values were observed among the two subclades of *O.* cf. *ovata* and *Ostreopsis* sp. 7 (ANOVA, $p > 0.05$), whereas their DV, W and average DV/W ratio values were significantly different from those of *Ostreopsis* sp. 6 (ANOVA, $p < 0.05$).
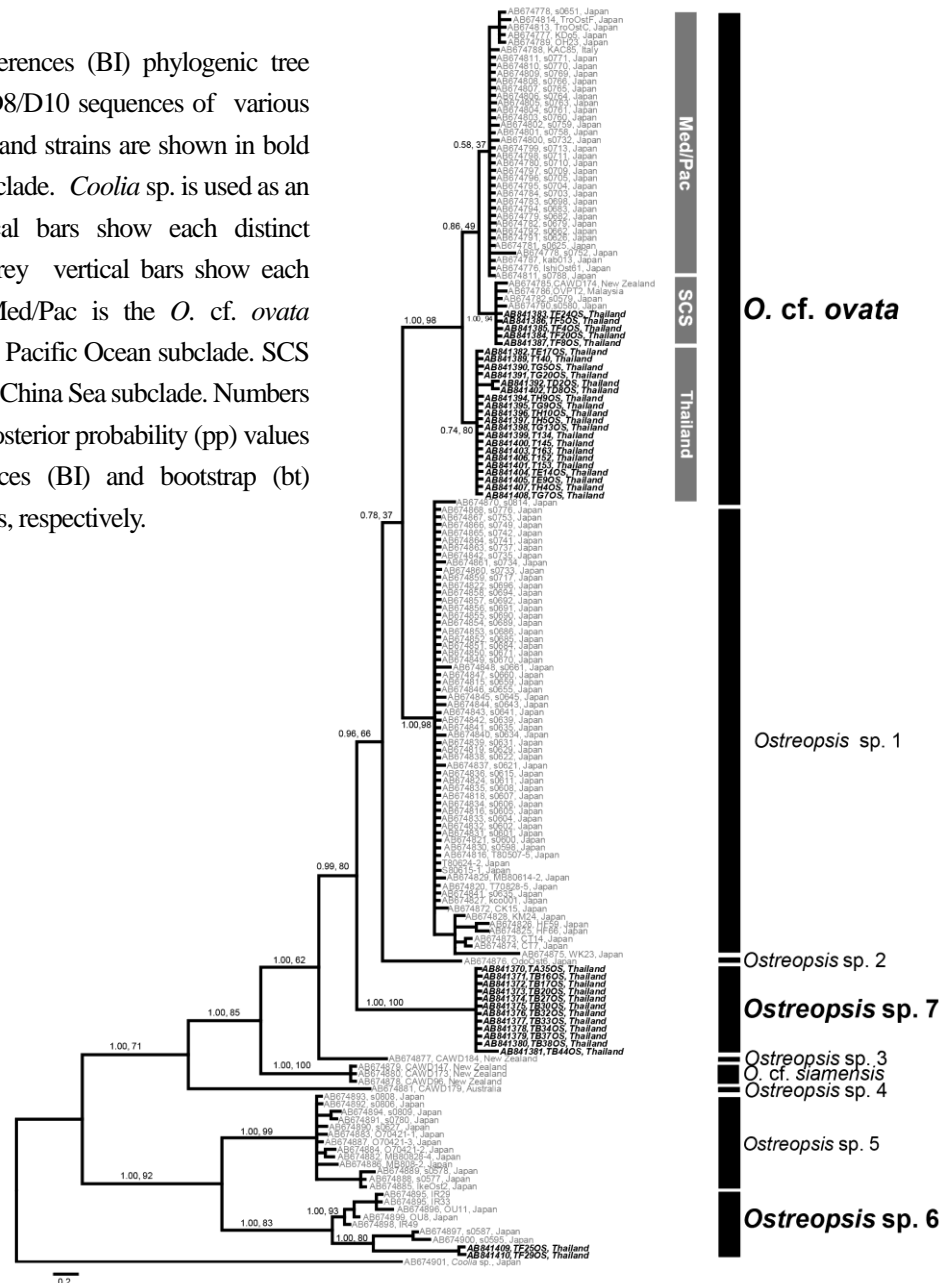
The SEM analyses showed morphologies of *Ostreopsis* sp. 7 cells exhibited a smooth thecal surface covered with spaced round pores, and 0.20–0.34 μm in diameter (average: 0.30±0.03 μm). The apical pore (Po), with an average length of 8.0 μm, was short and slightly curved. No statistically significant differences in dorso-ventral diameter (DV), trans-diameter (W) and DV/W ratio values were observed among *Ostreopsis* sp. 7 and the two subclades of *O.* cf. *ovata* (ANOVA, $p > 0.05$). Morphometric information revealed that the DV and DV/W ratio in *Ostreopsis* sp. 7 were also smaller than that of *O. ovata*.

The phylogenetic analyses based on the large subunit rDNA (LSU rDNA D1/D2 and D8/D10) and internal transcribed spacer containing the 5.8S rDNA (ITS-5.8S rDNA) sequences of the *Ostreopsis* species from Thailand were also performed using Maximum likelihood (ML) and Bayesian Inference (BI) methods. With *Coolia* sp. as the outgroup, the LSU rDNA D8/D10 tree shows that there are nine distinct clades (*O.* cf. *ovata*, *O.* cf. *siamensis* and *Ostreopsis* spp. 1–7 clades) (Fig. 2). The phylogenetic trees revealed that strains of the *O.* cf. *ovata* clade were apparently divided into three subclades. One subclade included 36 strains from the Mediterranean Sea and the East Pacific Ocean (Med/Pac). Some strains from Thailand (TF24OS, TF5OS, TF4OS, TF20OS and TF8OS) belonged to *O.* cf. *ovata* South China Sea (SCS) subclade together with the strains from Malaysia (OVPT2), Japan (s0579 and s0580) as well as New Zealand (CAWD174) (Fig. 2). Twenty-two strains of the *O.* cf. *ovata* clade from Thailand formed a novel monophyletic subclade, named Thailand subclade (Fig. 2). The relationship of this subclade to the other two subclades previously described was moderately supported by two analyses (BI = 0.74, ML = 80). All strains contented in *Ostreopsis* sp.1 restricted to Japanese coast, with strong supported values (BI = 0.98, ML = 99).

Twelve strains from Thailand were fully supported as a novel clade (BI = 1.00, ML = 100), which did not belong to any known clade (Fig. 2). This clade was phylogenetically positioned between *Ostreopsis* sp. 2 (OdoOst6) and *Ostreopsis* sp. 3 (CAWD184), which was confirmed in two different analyses: BI = 0.99 and ML = 80. This clade was newly named as *Ostreopsis* sp. 7 following Sato et al. (2011) who previously designated the unidentified *Ostreopsis* clades from Japanese coasts as *Ostreopsis* sp. 1 to sp. 6 (Fig. 2). Furthermore, results of the LSU rDNA D1/D2 and ITS-5.8S rDNA trees also confirmed that the clade of *Ostreopsis* sp.7 was clearly

divergent from other known *Ostreopsis* clades (data not shown). *Ostreopsis* cf. *siamensis* (CAWD96, CAWD147 and CAWD173) and *Ostreopsis* sp. 4 (CAWD179) were recovered and placed respectively at the primitive position reported. In the phylogenies, *Ostreopsis* sp. 5 is a sister to *Ostreopsis* sp. 6 with the highest nodal support (BI = 1.00, ML = 92). In the *Ostreopsis* sp. 6 clade, two strains from Thailand (TF29OS and TF25OS) belonged to the clade containing strains (s0587 and s0595) from Japan (BI = 1.00, ML = 80) (Fig. 2).

Figure 2 Bayesian inferences (BI) phylogenic tree based on LSU rDNA D8/D10 sequences of various *Ostreopsis* strains. Thailand strains are shown in bold and italic letters in each clade. *Coolia* sp. is used as an outgroup. Black vertical bars show each distinct *Ostreopsis* clade and grey vertical bars show each *Ostreopsis* subclade. Med/Pac is the *O.* cf. *ovata* Mediterranean Sea and Pacific Ocean subclade. SCS is the *O.* cf. *ovata* South China Sea subclade. Numbers at nodes represent the posterior probability (pp) values from Bayesian inferences (BI) and bootstrap (bt) values from ML analyses, respectively.

The genus *Gambierdiscus* from Thailand

The morphology of *Gambierdiscus* strain TF9G from Thailand was observed using LM and SEM. The results show that the strain TF9G displayed the typical *Gambierdiscus* plate formula of Po, 4′, 0a, 6″, 6c, ?s, 5‴, 0p and 2⁗ (Fig. 3A–F). The observed morphological features and their associated morphometric information enabled the strain to be identified as *G. caribaeus*. The cells were round and anterior-posteriorly compressed in apical view (Fig. 3B and C). In the SEM micrographs of Thai strain, the depth (D), length (L) and width (W)

were 64.4 ± 6.1, 44.1 ± 6.7 and 62.9 ± 6.6 μm, respectively. The ratios for D to W and L to W in SEM micrographs were 1.0 ± 0.1 and 0.73 ± 0.08, respectively. The 1′ plate was the smallest epithecal plate, while the 2′ plate was rectangular and the largest epithecal plate (Fig. 3A–C). The ratios of 2′/1″ to 2′/3″ plate suture lengths obtained from LM and SEM were 0.91 ± 0.12 and 0.95 ± 0.12, respectively. The 3′ plate was irregular pentagonal and the 4′ plate was hexagonal. The trapezium-shaped 6″ plate was very small. The 1″ plate was pentagonal and in contact with the 1′, 2′, 4′ and 2″ plates. The 2″ plate was quadrangular and the largest precingular plates. The 3″ plate was almost symmetry with an average ratio of 0.94 ± 0.16 between 3″/2″ to 3″/4″ plate suture lengths. The 4″ plate was quadrangular and the 5″ plate was pentagonal. The six cingular plates of strain TF9G are shown in Fig. 3D. The pentagonal 2‴′ plate was the largest in the hypotheca. The triangular 1‴ and quadrangular 5‴ plates were smaller than 2‴ and 4‴ plates. The 3‴ plate was quadrangular. The surface of the cells was smooth with many round or oval pores. The Po, which was located at the center of the cell, was elliptical and had a large fishhook-shaped opening with an average of 8.8 ± 0.1 μm in length and 6.7 ± 0.1 μm in width surrounded by 37 ± 6.8 pores (Fig. 3F).
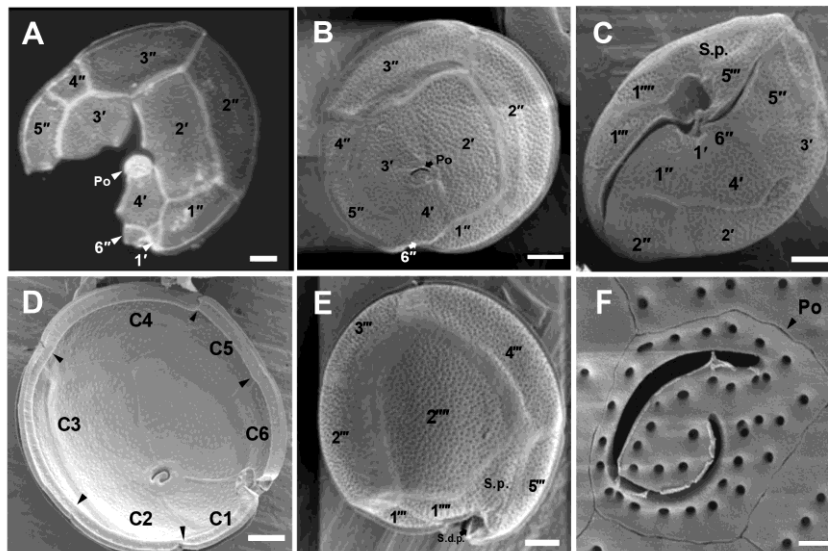

Figure 3 Morphology of *G. caribaeus* (strain TF9G) with LM-epifluorescence (A), SEM (C–F). A : Epithecal view. B: Epithecal view. C: Cell in ventral sulcal view. D: The six cingular plates shown within the epitheca. E: Hypothecal view. F: Elliptical apical pore (Po) plate with pores and a large fish hook-shaped opening. Scale bars = 10 μm (A–E) or 1 μm (F).

Three new LSU rDNA D8/D10 sequences of *Gambierdiscus* spp. from Thailand were obtained. The phylogenetic analyses based on the LSU rDNA D8/D10 sequences indicated that three *Gambierdiscus* stains from Thailand (TF9G, TF26G from the Gulf of Thailand and PG from the Andaman Sea) were well fitted with *G. caribaeus* clade formed by previous strains from Caribbean Sea strains (BZ08087_1, NOAA13_9, NOAA21_5, NOAA14_8, NOAA11_1, NOAA20_5 and NOAA19_1) and from Pacific Ocean strains (NOAA10_6 and NOAA7_2). The relationship of *G. caribaeus* from Thailand was supported by supported values from two analyses (BI = 1.00, ML = 86) (Fig. 4).
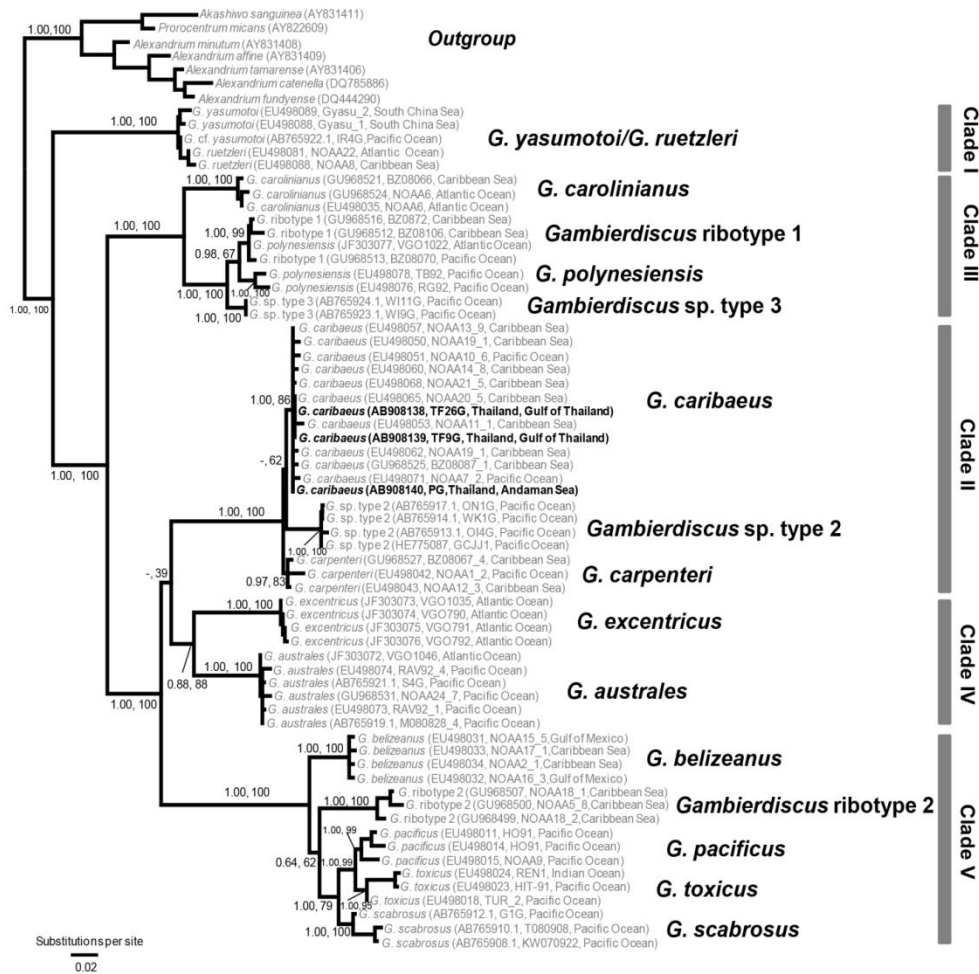
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Figure 4 Maximum likelihood (ML) phylogenic tree based on LSU rDNA D8/D10 sequences from various *Gambierdiscus* strains. *Akashiwo sanguinea*, *Prorocentrum micans*, *Alexandrium minutum*, *A. affine*, *A. tamarense*, *A. catenella* and *A. fundyense* were used as outgroup in the *Gambierdiscus* phylogeny. The bold lettering next to taxon names mark new sequences obtained in this study. Numbers at nodes represent the posterior probability (pp) values from Bayesian inferences (BI) and bootstrap (bt) values from ML analyses, respectively.

The genus *Coolia* from Thailand

Eight strains (PC2, PC3, TA20C, TD4C, TD6C, TF20C, TF40C and TH12C) of *Coolia* from Thailand were investigated their morphologies using SEM observation. The overall cell sizes of the strains observed were ranged for 19.6–33.6 µm in length and 19.4–33.0 µm in width. All Thai *Coolia* strains observed from Thailand showed a plate formula of Po, 4′, 0a, 6″,?c, ?s, 5‴, 0p and 2⁗. In the lateral view, the Thai *Coolia* strains were spherical in dorso-ventral view and ellipsoid (Fig. 5A). The shape of the 3′ of most cells was pentagonal and was in contact with the APC, 2′, 4′, 4″, 5″ and 3″ plates (Fig. 5B and F). The 4′ plate was narrow and oblong. The right margin of 4′ plate was straight touching the 5″ plate (Fig. 5B). The quadrangular 4″ did not touch the hexagonal 4′ plate and the 5″ plate was the biggest epithecal plate (Fig. 5E). The 6″ plate was small with the 0.84–1.75 of the W to L ratio. The triangular 1‴ plate was smaller than the 2‴, 3‴, 4‴ and 5‴ plates. Almost Thai *Coolia* strains showed that the 3‴ plate was much larger than the 4‴ plate and occupied the central part of the hypotheca (Fig.

5E). The 2‴″ plate of these cells was quadrangular, touching the S.p., 3‴, 4‴, 5‴ and 1‴″ plates, but not touching the 2‴ plate. In SEM micrographs of eight *Coolia* strains, the short and slightly curved apical pore complex (APC) was positioned dorsally and touches the 1′, 3′, 4′, 2″ and 3″ plates (Fig. 5F and G). The length of the narrow apical pore located in the APC was approximately 5–6 μm. The theca surface was smooth, irregularly scattered with large round pores 0.30 μm in average diameter (Fig. 5G). Considering all the morphological features, we identified the Thai *Coolia* strains as *Coolia malayensis.*
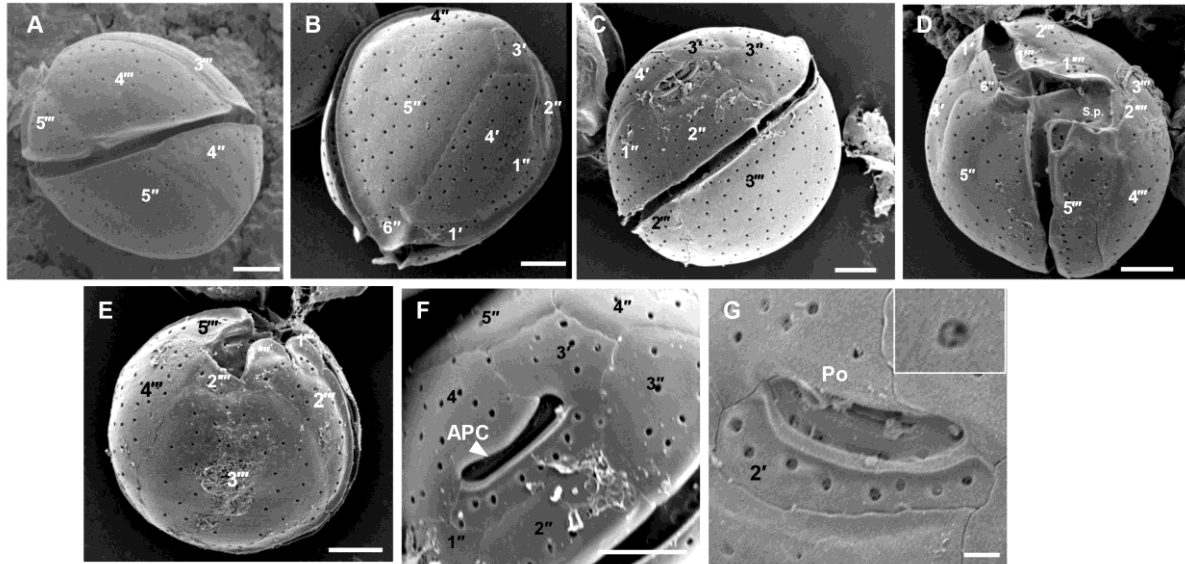

Figure 5 Morphology of *C. malayensis* (strain) TD6C with SEM (A–G). A : Cell in lateral view. B: Cell in apical view showing the epithecal plate pattern. C: Cell in dorsal view showing the epithecal and hypothecal plates. D: Ventral view showing the epithecal and hypothecal plates with wide cingulum and sulcus. E: Cell in antapical view showing the plate pattern. F: the 3′ plate and the adjacent plates. G: Apical pore complex (APC) and round theca pore showing small pore inside. Scale bars = 5 μm (A–F) or 1 μm (G).

Nineteen new LSU rDNA D1/D2 sequences of Thai *Coolia*, were obtained from Thai waters. The phylogenetic trees showed four distinct species: *C. canariesensis*, *C. tropicalis*, *C. monotis* and *C. malayensis*, with the robust supported values from BI and ML analyses. Eighteen *Coolia* strains isolated from the Gulf of Thailand (T60C, T73C, TD4C, TD5C, TD6C, TF20C, TF27C, TE18C, TE20C TF40C and TH12C) and the Andaman Sea (P1C, P2C, P3C, TA20C, TA23C, TA24C and TC9C) were clustered with *C. malayensis* clade comprised of four previous strains from Malaysia (CmPL01), Korea (CMJJ2), Caribbean Sea (CCMP1345) and New Zealand (CAWD39 identified as *C. monotis*). The relationship of *C. malayensis* from Thailand was supported by high nodal supports from two analyses (BI = 0.92, ML = 93) (Fig. 6). Furthermore, some of Thai *Coolia* strain (CT1C1 from the Gulf of Thailand) belonged to *C. tropicalis* clade together with two strains from Indonesia (VG0923) and from Caribbean Sea (CCMP1744), with the robust support value from two analyses (BI = 1.00, ML = 100). In this study, we were not able to identify morphology of the CT1C1 strain from Thailand, which belonged to a clade of *C. tropicalis* described in the results of phylogenetic analyses because we lost the culture.
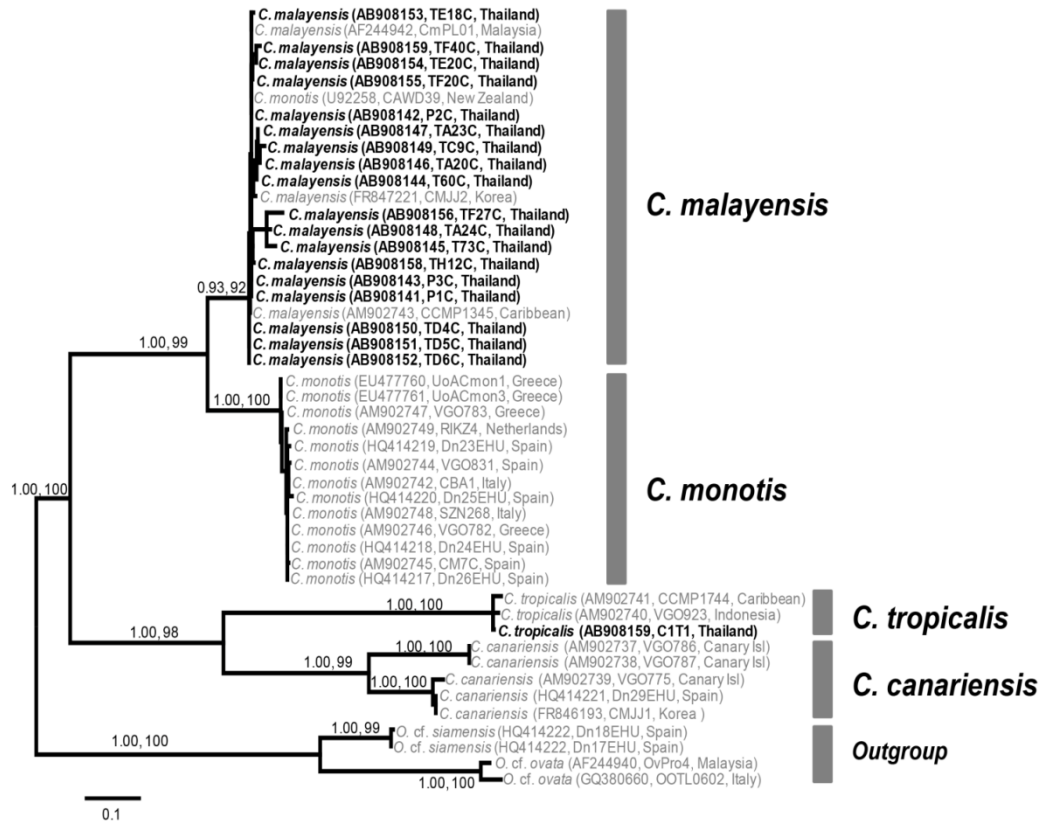
Figure 6 Maximum likelihood (ML) phylogenic tree based on LSU rDNA D1/D2 sequences from various *Coolia* strains. *Ostreopsis ovata* and *O. siamensis* were used as outgroup in the *Coolia* phylogeny. The bold lettering next to taxon names mark new sequences obtained in this study. Numbers at nodes represent the posterior probability (pp) values from Bayesian inferences (BI) and bootstrap (bt) values from ML analyses, respectively.

Distribution of benthic dinoflagellates in Thailand

The macroalgal samples were collected along the coasts of the Gulf of Thailand and the Andaman Sea (Fig. 7). During sampling in the Gulf of Thailand and the Andaman Sea, the temperature (27–31˚C) and salinity (27–34) of the seawater were not different. In the present study, the cell densities of *Ostreopsis* spp., *Gambieridiscus* spp. and *Coolia* spp. were 0.140–174, 0.050–3.230, 0.200-47.040 cells/g wet macroalgae, respectively. The highest abundance of *Ostreopsis* cells (174 cells/g wet macroalgae) were obtained in samples from Ao Khung Wiman (TH) whereas those of *Gambierdiscus* (3.230 cells/g wet macroalgae) and *Coolia* (47.040 cells/g wet macroalgae) cells were obtained in samples from Ao Tung Khen (P) (Fig. 7).

In this study, *O.* cf. *ovata* was the dominant benthic dinoflagellate species in Thailand. In the Gulf of Thailand, *O.* cf. *ovata* Thailand subclade appeared at all localities (Fig. 7) except for Koh Wai (TF) where *O.* cf. *ovata* belongs to the South China Sea subclade and *Ostreopsis* sp. 6 were found in this sampling point. Fourteen strains belonging to *Ostreopsis* sp. 7 were found at both localities (Hat Thai Mueang: TA and Khao Lak: TB) on the Andaman Sea coast, while no strains of *Ostreopsis* sp. 7 were found in the Gulf of Thailand (Fig. 7). *Gambierdiscus caribeaus* were found at Koh Wai (TF) in the Gulf of Thailand and at Ao Tung Khen (P) in the Andaman Sea (Fig. 7). All sampling sites in both the Gulf of Thailand and the Andaman Sea were found *C. malayensis*, whereas a strain placed in a clade of *C. tropicalis* was found at Koh Raed (CT) in the Gulf of
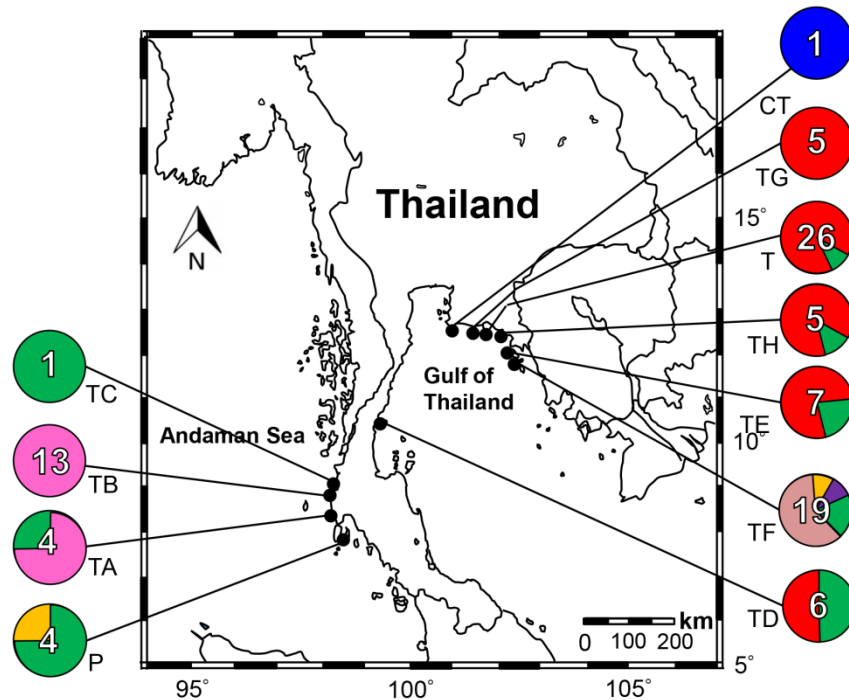
Thailand (Fig. 7).

Figure 7 Geographic distributions of *Ostreopsis*, *Gambierdiscus* and *Coolia* plotted on map showing Gulf of Thailand and the Andaman Sea coasts of Thailand. Pie charts illustrate species/clade/subclade composition of each sample; total number of strains is indicated. Each color corresponds to species/clade/subclade: red, *O.* cf. *ovata* Thailand subclade; light red, *O.* cf. *ovata* South China Sea subclade; purple, *Ostreopsis* sp. 6; pink, *Ostreopsis* sp. 7; orange, *G. caribaeus*; green, *C. malayensis* and blue, *C. tropicalis*. Numbers of strains are shown inside pie chart. Each location is indicated by the following code as shown in Fig. 1: CT, Koh Raed, Chonburi; P, Ao Tang Khen, Phuket ; T, Koh Man, Rayong; TA, Hat Thai Mueang, Phang-Nga; TB, Khao Lak, Phang-Nga; TC, Leam Pagarung, Phang Nga; TD, Hat Sai Ri, Chumphon; TF, Koh Wai, Trat; TE, Koh Chang, Trat; TG, Khao Leam Ya, Rayong; TH, Ao Khung Wiman, Chanthaburi.

In conclusion, the benthic dinoflagellates *O.* cf. *ovata*, *Ostreopsis* sp. 6, *Ostreopsis* sp. 7, *G. caribaeus*, *C. malayensis* and *C. tropicalis* were identified from Thailand. These results suggest that *Ostreopsis* sp. 7 is distributed in the Andaman Sea, whereas the two subclades of *O.* cf. *ovata*, *Ostreopsis* sp. 6 and *C. tropicalis* are distributed in the Gulf of Thailand. *Gambierdiscus caribaeus* and *C. malayensis* were found not only in the Gulf of Thailand but also in the Andaman Sea.

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