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Seasonal distribution of *Gambierdiscus* spp. in Wakasa Bay, the Sea of Japan, and antagonistic relationships with epiphytic pennate diatoms

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Abstract

The occurrence of the ciguatera fish poisoning (CFP) causative *Gambierdiscus* spp. was confirmed in the Sea of Japan for the first time in 2009. This paper reports seasonal distribution of *Gambierdiscus* spp. and epiphytic diatoms in the Sea of Japan. Monitoring results suggested an antagonistic interaction in abundances between epiphytic diatoms and the dinoflagellate *Gambierdiscus* spp. Allelopathic effects of diatoms were considered to be involved in the competitive phenomenon. Therefore it is hypothesized that cell densities of epiphytic pennate diatoms on macroalgae are a novel determinant affecting the abundance of *Gambierdiscus* spp. other than sea water temperature, salinity and nutrients. Monitorings of the abundance of epiphytic diatoms would lead us to predict the occurrences of *Gambierdiscus* spp. blooms in the CFP area, and thereby the CFP risk assessments would be developed. Phylogenetic analyses indicated that *Gambierdiscus* spp. in the Sea of Japan belonged to be *Gambierdiscus* sp. type 2 which was reported to be non-toxic. Nevertheless, based on morphological characteristics, at least two types of *Gambierdiscus* spp. were found in the Sea of Japan. It is needed to test the toxicity of the both types of *Gambierdiscus* recognized in the present study for evaluation of the probability of CFP outbreak risks in the Sea of Japan in the future.

1. Introduction

Ciguatera fish poisoning (CFP) is one of the most significant marine food-borne illness caused by the consumption of large-sized fish and reef fish in the tropical and subtropical sea, and affects 25,000 – 500,000 people annually around the world (Fleming et al., 1998; Lehane and Lewis, 2000; Yasumoto, 2005; Berdalet et al., 2017). The symptoms are characterized by gastrointestinal, neurological and cardiovascular disturbances, and the most characteristic symptom is the dysesthesia called “Dry Ice Sensation”. The major toxins of CFP are ciguatoxins (CTXs) and maitotoxins (MTXs). CTXs and MTXs are produced primarily by dinoflagellate species in the genus *Gambierdiscus* attached to macroalgae. Herbivorous fishes ingest *Gambierdiscus* cells attached to macroalgae. Furthermore, large-sized fish-eating fishes prey the herbivorous fishes and turn them to be poisonous. The consumption of strongly toxin-contaminated fishes via biological concentration causes poisoning and illness.

Gambierdiscus toxicus, which is the type species of the genus *Gambierdiscus*, was discovered in the Gambier Islands, French Polynesia in the late 1970’s (Adachi and Fukuyo, 1979). To date, *Gambierdiscus* species were found to be widely distributed throughout the tropical and subtropical seas in the world such as French Polynesia, the Caribbean Sea, Hawaiian Islands, Australia, and the Indian Ocean. In Japan, different phylotypes of *Gambierdiscus* sp. Type 1 (i.e.,= *G. scabrosus*) (Kuno et al., 2010; Nishimura et al., 2013, 2014), *Gambierdiscus* sp. type 2 (Kuno et al., 2010; Nishimura et al., 2013), *Gambierdiscus* sp. type 3 (Nishimura et al., 2013), *G. australes* (Nishimura et al., 2013), and *G.* cf. *yasumotoi* (i.e.,= *Fukuyoa* cf. *yasumotoi*) (Nishimura et al., 2013; Gómez et al., 2015) were reported based on the sequence analyses of D8-D10 region of the nuclear large subunit (LSU) ribosomal DNA (LSU rDNA D8-D10) and the nuclear small subunit (SSU) rDNA.

CFP has occurred in Nansei Islands in the subtropical region in Japan and the distribution of ‘*G. toxicus*’ has been investigated. In the main island of Japan, CFP has occurred sporadically in the temperate area of the Pacific coast (Oshiro et al., 2010; Toda et al., 2012). Moreover, the detections of ‘*G. toxicus*’ have been reported from the subtropical to temperate areas in the Pacific coasts (Hara and Horiguchi, 1982; Koike et al., 1991;

Ishikawa and Kurashima, 2010). Although the existence of CFP has never been reported before in the Sea of Japan, the inhabitation of *Gambierdiscus* spp. was confirmed for the first time in Wakasa Bay in 2009 (Hatayama et al., 2011). The maximum cell density of *Gambierdiscus* spp. in the Sea of Japan at that time was almost the same level with that of Okinawa where CFP had actually occurred (Koike et al., 1991; Hatayama et al., 2011). Two *Gambierdiscus* spp. strains in the Sea of Japan were found to be *Gambierdiscus* sp. type 2, which was suggested to be non-toxic. On the other hand, *Gambierdiscus* sp. type 2 was reported to be dominant in the temperate area in the Pacific coast where CFP outbreaks had been reported (Nishimura et al., 2013). Nevertheless, in addition to *Gambierdiscus* sp. type 2, toxic species (*Gambierdiscus* sp. type 3, *G. scabrosus* and *G. australes*) were also found in the temperate area in the Pacific coast (Nishimura et al., 2013, 2014). Therefore, there is a possibility of the existence of toxic species in the Sea of Japan.

In the present study, the authors monitored *Gambierdiscus* spp. in order to assess the risk of CFP events in the Sea of Japan and investigated broad distribution in the coasts of the Sea of Japan and morphological and phylogenetic characteristics.

2. Materials & Methods

2.1. Samplings and measurements of environmental factors

Collections of macroalgae samples were carried out by snorkeling in summer and by raking using a special instrument (Shoei Igaitoriki chu No.548 – 1) in winter, during the periods from March 2010 to March 2011 and from July to October 2011, respectively, in Niizaki, Ine town, Kyoto Prefecture (0.5 - 4.0 m depth, 35° 41.52’ N, 135° 18.28’ E) (Fig. 1). Collected macroalgae samples were 6 species of Chlorophyceae, 22 species of Phaeophyceae, 23 species of Rhodophyceae and one species of *Liliopsida* during the entire investigation. In case of snorkeling, samples were placed into zipper plastic bags in the sea and put into polyethylene bottles on land, and in case of raking, samples were directly taken into 1L polyethylene bottles on land. Samples in polyethylene bottles were kept in a cool

box on ice and brought back to the laboratory. In the field, the temperature and salinity of sea water were simultaneously measured using a water-temperature-salt-measuring-instrument (Kent, EIL5005) along with samplings. In October 2010, samples were similarly collected at three points of Noto Peninsula: Ogi (0 – 0.5 m depth, 37° 18.11’ N, 137° 14.21’ E), Rokko Cape (0.5 – 1.5 m depth, 37° 31.43’ N, 137° 19.41’ E) and Monzen (0 – 0.5 m depth, 37° 17.31’ N, 136° 43.42’ E) in Ishikawa Prefecture, and at two points of Oshima Peninsula: Yoshioka fishery harbor (1.5 – 2.5 m depth, 41° 26.35’ N, 140° 14.17’ E) and Yagoshi Point (2.0 – 4.0 m depth, 41° 31.26’ N, 140° 24.58’ E) in Hokkaido for a broad distribution research (Fig. 1).

2.2. Sample treatments and cell observation / count

Algal sample treatments, observations and counts of epiphytic microalgal cells were described in the previous study by Ishikawa and Kurashima (2010). Briefly, after adding filtrated seawater up to 1L in the polyethylene bottles with samples, the bottles were sealed up. Subsequently the periphytic microalgae were detached by vigorously shaking the bottles 200 times. After shaking, the suspensions were filtrated serially through sieves of 200 and 20 μm meshes. Neutralized formaldehyde was added to the collected suspension of residue retained on the 20 μm sieve to fix cells with a final concentration of 1% (v/v). The residue materials retained on the 200 μm sieve were centrifuged manually by a salad spinner to remove extra seawater attached to the materials, and the wet weights were measured. For microscopic observations, Calcofluor white M2R (1 mg/mL, Sigma-Aldrich Co, St. Louis, MO, USA) solution was added to samples to stain thecae of the dinoflagellates with a final concentration of 10 μg per mL. Samples were settled for over 30 minutes and the thecae of dinoflagellate cells in samples were well stained (Fritz and Triemer, 2004). Dinoflagellate cells in samples were observed under UV light excitation (350 nm, EX330-380, UV-2A, BA420) using an inverted epifluorescence microscope (Nikon, TE300). *Gambierdiscus* spp. cells were counted 3 times in each sample (0.5 mL) and cell density was determined. Cells of 1p narrow type *Gambierdiscus* spp. were counted as described in Litaker et al., (2009) and the rate of 1p narrow type cells in all

Gambierdiscus spp. cells was measured. In case of the red alga *Amphiroa zonata* samples, epiphytic diatom cells were counted 3 times in 0.05 mL per each sample using a light microscope and cell density was also obtained.

2.3. Single-cell polymerase chain reaction (PCR) and sequencing

Single cells of *Gambierdiscus* spp. were isolated from field samples before the fixing with neutralized formaldehyde, using capillary without contamination of other microalgal cells. Each cell was placed into a PCR tube immediately and stored at -80°C until processing. GoTaq® Green Master Mix (Promega) was used for PCR. The D8-D10 region of LSU rDNA was amplified using D8F (5'-CGGGAAAAGGATTGGCTCT-3') and D10R (5'-ATAGGAAGAGCCGACATCG-3') primers, which is described in Litaker et al. (2010). The reaction mixture contained 25 µl of 2 × GoTaq Green Master Mix, 1 µl of D8F primer (10 µM), 1 µl of D10R primer (10 µM) in a total volume of 50 µl. The mixture was added into the PCR tube with a cell. The tube was set to a thermal cycler (Biorad) and the DNA was amplified. The PCR was carried out using the reaction conditions as follows: an initial denaturation at 95°C for 2 min; followed by 35 cycles: 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min; and the extension at 72°C for 5 min. The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Sequencing was analyzed at the Dragon Genomics Center (Takara bio). The obtained sequence data were edited by chlomas lite (Technelysium Pty Ltd 2005) and the DNA sequences were determined.

2.4. Phylogenetic analysis of *Gambierdiscus* spp. in Niizaki

DNA sequences obtained from *Gambierdiscus* spp. cells were determined by using Basic Local Alignment Search Tool (BLAST) that is a homology search software in National Center for Biotechnology Information (NCBI). The present sequences having the strongest homology with those of *Gambierdiscus* LSU rDNA D8-D10 were determined as *Gambierdiscus* spp.

Phylogenetic trees were made using *Gambierdiscus* spp. LSU rDNA D8-D10

sequences obtained in the present study and some *Gambierdiscus* LSU rDNA D8-D10 sequences registered in NCBI. Reconstruction of the maximum likelihood (ML) trees of the D8-D10 region of the LSU rDNA from *Gambierdiscus* spiecies/phylotypes and ML bootstrap (BP) calculations (1000 replicates) were carried out using the program MEGA 5.0 (Tamura et al., 2011) with the GTR model incorporating invariable sites and a discrete gamma distribution (GTR + I + G). Additionally, *p*-distances were calculated using the program MEGA 5.0 (Tamura et al., 2011).

3. Results & Disccusion

3.1. Seasonal changes of *Gambierdiscus* spp. and epiphytic diatoms on macroalgae in the Sea of Japan and broad distribution

During the widespread investigation, *Gambierdiscus* spp. had been detected at the sites of Niizaki and Inechou, Kyoto Prefecture. The maximum cell density of *Gambierdiscus* spp. was 262 cells per g wet weight (ww) in *Marginisporum crassissimum* in October 2010. On the other hand, *Gambierdiscus* spp. cells were not detected at the three investigation sites of Noto Peninsula and two sites of Oshima Peninsula (Fig. 1).

Since the macroalgae consistently collected during the entire investigation term was the red alga *Amphiroa zonata*, cell densities of *Gambierdiscus* spp. and epiphytic diatoms were measured together with environmental factors (sea water temperature and salinity). The authors found that *Gambierdiscus* spp. cells (2.0 cells per g ww) in July 2010 and the cell density decreased in August 2010 again (Fig. 2). And then, *Gambierdiscus* spp. showed rapid increase until December 2010. The maximal cell density of *Gambierdiscus* spp. (253 cells per g ww) was recorded in December 2010 (Fig. 2). Nevertheless, they continued dramatically reducing since January 2011 and were not detected in March 2011 (Fig. 2). In 2011, *Gambierdiscus* spp. cells were found with a low density (0.15 cells per g ww) in July when the observation was restarted (Fig. 2). Then it increased sharply in

August reaching a high density (115 cells per g ww). In September *Gambierdiscus* spp. decreased rapidly and its cell density in October also showed low values (0.57 – 1.5 cells per g ww) (Fig. 2).

When the sea water temperature was low in winter and spring, cell densities of *Gambierdiscus* spp. tended to be quite low (Fig. 2). Therefore the sea water temperature appears to give the largest influence on seasonal changes of *Gambierdiscus* spp. There was little difference in sea water temperatures between that ascending in May 2010 and that descending in November – December 2010. Nevertheless, the cell densities of *Gambierdiscus* spp. in November – December 2010 were by far higher than that in May 2010 (Fig. 2). The data suggest that most of *Gambierdiscus* spp. cells, which have increased under comparatively warm sea water temperature in August – November 2010, survived and they had grown until the sea water temperature decreased to the level of 15°C. It is supposed that *Gambierdiscus* spp. decreased dramatically due to a rapid decrease in the sea water temperature more than 4°C between December 2010 and January 2011 because it gave an enormous influence on the dinoflagellates.

Pennate diatoms *Amphora* spp. and *Naviculoid* diatoms were observed as dominant epiphytes during the entire investigation period (Fig. 3). The third and fourth abundant taxa were *Meuniera* spp. and *Amphora hyalina*, respectively. Nevertheless, *Nitzschia longissima* was the most dominant species in August 2010 with cell density of 5,404 cells per g ww. Moreover, the cell density of *Triceratium* spp. was found to be higher than that of any other diatoms in June 2010 (cell density: 6,074 cells per g ww). Nevertheless, *Amphora* spp. and *Naviculoid* diatoms were usually detected at high densities in almost the entire period of survey, and largely contributed to the seasonal changes of all diatoms. The maximum cell density of *Amphora* spp. was observed in September 2011 (197,800 cells per g ww) and the average density of *Amphora* spp. was 21,640 cells per g ww. The maximum and mean cell densities of *Naviculoid* diatoms were 107,400 cells per g ww in February and 13,970 cells per g ww, respectively (Fig. 3).

The contributing factor to increase or decrease in *Gambierdiscus* spp. from July to September 2011 were not clear based on the influence of sea water temperature. Although the cell density of epiphytic diatoms was low (< 10,000 cells per g ww) in July 2011, it

drastically increased (> 200,000 cells per g ww) in September 2011 (Fig. 3). These results indicate that the surface areas on macroalgae occupied by diatoms went blank and in turn *Gambierdiscus* spp. could find attaching areas. Nevertheless, it is presumed that very high cell density (> 200,000 cells per g ww) of diatoms (mostly *Amphora* spp.) in September 2011 caused a dramatic decrease in the surface areas where *Gambierdiscus* spp. could attach and thus the cell density of *Gambierdiscus* spp. had decreased again (Fig. 3). Considering cell densities of diatoms, the increases or decreases in *Gambierdiscus* spp. cells from July to December 2010 probably occurred by the relatively low densities of attached diatoms (Fig. 3). An antagonistic interaction was also observed in abundances between epiphytic diatoms and the dinoflagellates (Fig. 4). An interaction mediated by growth inhibiting chemicals (i.e. allelopathy) is one of the important factors of phytoplankton species shifts in the sea (Prince et al., 2008; Poulson-Ellestad et al., 2014). Information has been explosively accumulated for allelopathy of phytoplankton, which has been reported in many different taxonomic groups such as cyanobacteria, diatoms, dinoflagellates, haptophytes and raphidophytes (Granéli et al., 2008). For example, it was described that the benthic diatom *Amphiprora hyaline* inhibits the growth of a *Chattonella* (*Chattonella antiqua*) (Miyashita et al., 1994). In addition, there is an antagonistic interaction for population dynamics between the red tide raphidophyte *Heterosigma akashiwo* and the diatom *Skeletonema* spp. in marine coastal ecosystems (Pratt, 1966). Moreover it was suggested that the allelopathy plays an important role in predominance of *Gambierdiscus* spp. on macroalgae (Bomber et al., 1988). Thus, it can be assumed that the similar competitive phenomenon by allelopathy affect population dynamics between diatoms (mainly *Amphora* spp.) and *Gambierdiscus* spp. A sudden increase in diatoms in September 2011 would be attributable to the massive nutrient supply by increased inflow of southern vicinity of Yura River in September 20 to 23 (Fig. 5) (Ministry of Land, Infrastructure, Transport and Tourism: http://www1.river.go.jp/cgi-bin/DspWaterData.exe?KIND=2&ID=306091286605080&BGNDATE=20110901&ENDDATE=20121231&KAWABOU=NO). A decrease in salinity was observed at Niizaki in September 2011 by inflow of Yura River water described above. It is presumed that an increase in diatoms by the massive nutrient supply caused a decrease

in *Gambierdiscus* spp. The salinity range was optimal or semi-optimal for *Gambierdiscus* spp. as described in Yoshimatsu et al. (2014) during the entire investigation period (Fig. 5), and nutrient concentrations appeared to show little correlation with the growth of *Gambierdiscus* spp. (Yasumoto et al., 1980).

Gambierdiscus spp. cells were not detected at the investigation sites other than Wakasa Bay (Niizaki). Sea water temperature (18 to 19°C, Japan Meteorological Agency: http://www.jma.go.jp/jma/menu/report.html) and salinity at other sites (Noto Peninsula and Oshima Peninsula) at the sampling date were in the adequate range for the growth of *Gambierdiscus* spp. (Parsons et al., 2012). One of the possible reason is the sea water temperature in winter. Sea water temperatures were below 10°C in Noto Peninsula and Oshima Peninsula during winter season (Japan Meteorological Agency: http://www.jma.go.jp/jma/menu/report.html). There is a possibility that *Gambierdiscus* spp. cannot survive and overwinter below 10°C (Yoshimatsu et al., 2014).

3.2. Morphology and phylogenetic analysis of *Gambierdiscus* spp. in the Sea of Japan

As a result of microscopic observation, most of *Gambierdiscus* spp. cells appeared to be a broad 1p type on the basis of the reports by Litaker et al. (2009) (Fig. 6A). The depth of 1p broad type cells was 67.5–105 µm (average 86.2 µm), while the width and length were 70.0–110 µm (average 84.9 µm) and 32.5–62.5 µm (average 50.8 µm), respectively. Furthermore, it was found that the total number of 1p narrow type cells was 32 from September to December 2010 (Fig. 6B). The ratio of 1p narrow type was 1.8 % (32 cells/1,731 cells) among the total cells of *Gambierdiscus* spp. in Niizaki, and 3.7 % (8 cells/212 cells) among the red alga *Amphiroa anceps* in November 2010 when 1p narrow type cells were found to be most abundant. The depth of 1p narrow type cells was 67.5–75.0 µm (average 72.5 µm), while the width and length were 65.0–82.5 µm (average 71.7 µm) and 40.0–47.5 µm (average 45.6 µm), respectively. It was significantly different in the depth, length and width between 1p broad and narrow type cells of *Gambierdiscus* spp. in Niizaki (depth: $t = 5.2$, $P < 0.001$, width: $t = 4.8$, $P < 0.001$, length: $t = 2.2$, $P < 0.05$). To

classify *Gambierdiscus* spp. according to the morphology, at least two types of *Gambierdiscus* spp. were noticed in Niizaki (Fig. 6).

The phylogenetic tree of *Gambierdiscus* species/phylotypes is presented in Fig. 7. The genetic distances indicated that the LSU rDNA D8-D10 sequences of all samples were 0–0.1 % and 0 % different from those of *Gambierdiscus* sp. type 2 strains and *G. caribaeus* GCJJ1 strain in Korea, respectively, and 2.5–3.1 % and 2.4–3.1 % different from those of the other *G. caribaeus* and *G. carpenteri* strains. In the ML phylogenetic tree inferred from the LSU rDNA D8-D10, all of the sequences from *Gambierdiscus* spp. in Niizaki obtained in the present study formed the same clade with *Gambierdiscus* sp. type 2 (Fig. 7) (Kuno et al., 2010; Nishimura et al., 2013). Although all of the sequences formed the same clade with *Gambierdiscus* sp. type 2 and the Korean strain of *G. caribaeus* GCJJ1, they formed a different clade with *G. caribaeus* and *G. carpenteri* (Fig. 7). This result shows that *Gambierdiscus* sp. in the Sea of Japan belongs to *Gambierdiscus* sp. type 2 along with Nishimura et al. (2013). The Korean strain GCJJ1 was reported to be *G. caribaeus* (Jeong et al., 2012). Since the Korean strain GCJJ1 belongs to the same clade with *Gambierdiscus* sp. type 2, it is considered that the Korean strain GCJJ1 should belong to *Gambierdiscus* sp. type 2 rather than *G. caribaeus* (Fig. 7).

Increased water temperatures in the Sea of Japan have been considered to significantly affect the expanding distribution of *Gambierdiscus* spp. to the Sea of Japan (Hatayama et al., 2011). Since floating macroalgae from the region such as Okinawa where *Gambierdiscus* spp. commonly inhabit have been transported into the Sea of Japan by Tsushima Current (Komatsu and Sugimoto, 2004), it is assumed that *Gambierdiscus* spp. also have been transported into the Sea of Japan together with the macroalgae. On the other hand, it is thought that *Gambierdiscus* spp. was not able to colonize in the Sea of Japan because of too low winter water temperature in the past. Nevertheless, at present, *Gambierdiscus* spp. can live and overwinter in the Sea of Japan owing to the increased water temperatures, especially in the winter season (Japan Meteorological Agency: http://www.jma.go.jp/jma/en/Activities/cc.html). The present study revealed that *Gambierdiscus* sp. in Niizaki was identified as *Gambierdiscus* sp. type 2. Therefore it is indicated that *Gambierdiscus* sp. type 2 is an endemic species, which have been living from

the north of East China Sea to the west of Japanese coastal waters including the Sea of Japan and the Pacific Ocean. Since both *G. caribaeus* and *G. carpenteri* were reported to live in a broad area of the Caribbean Sea and the Pacific Ocean (Litaker et al., 2010), it is suggested that these relative species including *Gambierdiscus* sp. type 2 are cosmopolitan. It remains to figure out the phylotype of morphologically different *Gambierdiscus* sp. in the Sea of Japan.

3.3. The existence of *Gambierdiscus* spp. and the risk of CFP in the Sea of Japan

The present study clarified that *Gambierdiscus* spp. was dominant in the coast of the Sea of Japan depending on living regions and seasons. The cell densities of *Gambierdiscus* spp. were 262 cells per g ww on the surface of the red alga *Marginisporum crassissimum* in October 2010 and 253 cells per g ww on the surface of the red alga *Amphiroa zonata*. These values were higher than any other values reported in the previous studies in Japan. The maximum cell density of *Gambierdiscus* spp. had been 51.0 cells per g ww on the surface of the red alga *Galaxaura* sp. collected in Akashima, Okinawa Prefecture (Koike et al., 1991). The period of emergence of the red alga *M. crassissimum* was short and it had been growing for a brief time. In fact, this alga was collected only once in October 2010. Therefore it is thought that the algal fronds were relatively new and *Gambierdiscus* spp. cells were able to attach to this alga better than diatoms. Then, *Gambierdiscus* spp. grew relatively quickly, and the maximal cell density was recorded. That is to say, *Gambierdiscus* spp. was able to attach to *M. crassissimum*, because it was less affected by the competition with diatoms. *A. zonata* had been observed for a long time in the year, and it is presumed that a competition between *Gambierdiscus* spp. and diatoms had already occurred on the surface of *A. zonata*, and various species of epiphytic diatoms fully attached to them.

Gambierdiscus spp. was found in high densities in the coast of Sea of Japan compared to the Pacific coast, and *Gambierdiscus* spp. in Niizaki belong to *Gambierdiscus*

sp. type 2. Moreover, Nishimura et al. (2013) reported that *Gambierdiscus* sp. type 2 was non-toxic species. There is a possibility of a negative correlation of toxin biosynthesis with active cell division (Chinain et al., 2010). Thus it is reasonable for *Gambierdiscus* sp. type 2 to enhance its growth rate rather than its toxicity, surviving in the temperate area where periods of optimum temperature are limited throughout the year.

In the present study, the authors found that there are at least two types of *Gambierdiscus* spp. in the Sea of Japan based on the characteristics of morphology. Single-cell PCR was carried out to detect genealogically different species, and only *Gambierdiscus* sp. type 2 could be obtained. Nishimura et al. (2013) reported that *Gambierdiscus* sp. type 2 was dominant in the temperate area. In addition to *Gambierdiscus* sp. type 2, *Gambierdiscus* sp. type 3, *G. scabrosus* and *G. australes* are also inhabiting in the temperate area in the Pacific coast (Nishimura et al., 2013, 2014). Although *Gambierdiscus* sp. type 2 was non-toxic species, *Gambierdiscus* sp. type 3, *G. scabrosus* and *G. australes* were toxic (Nishimura et al., 2013, 2014). Therefore there is a possibility that 1p narrow type *Gambierdiscus* sp. is toxic. Consequently, it is suggested in that case that toxic dinoflagellate-derived food-borne illness including CFP can break out in the Sea of Japan when toxic types become dominant. Thus toxicity tests for toxic dinoflagellates is needed in the Sea of Japan and the research for distribution of fish mediating poison are necessary. Moreover, a research for wide-area distribution of toxic dinoflagellates are also required throughout the coast of Sea of Japan and the monitoring is also essential at the point where their cell densities are high.

The limiting factor of the distribution of *Gambierdiscus* spp. were reported to be the sea water temperature, salinity and nutrients in the past (Parsons et al., 2012). The authors here propose cell densities of epiphytic diatoms as a new factor affecting the distribution of *Gambierdiscus* spp. In fact, it is reported that occurrences of harmful algal blooms are controlled by the cell densities of diatoms (Eppley 1977; Yamaguchi 1994; Shikata et al., 2010; Imai and Yamaguchi, 2012), and the possibility of control of red tides using harmless diatoms is suggested (Imai and Yamaguchi, 2012; Imai et al., 2017). Accordingly, investigations of field studies with frequent samplings and laboratory experiments would be essential to understand allelopathic interactions between

Gambierdiscus spp. and epiphytic diatoms on various macroalgae in the future. Since it is reported that epiphytic behavior (growth and attachment) is different among the *Gambierdiscus* species toward a variety of macroalgal hosts, it is preferable to evaluate multiple macroalgae (Parsons et al., 2011, Rains and Parsons, 2015).

Finally an importance of monitoring the cell densities of diatoms is proposed for prediction of the occurrences of *Gambierdiscus* spp. blooms in the area where CFP is common. And it is possible to enhance the probability of risk assessment of CFP. If the growth of epiphytic diatoms is promoted on the surface of macroalgae, the toxic blooms of *Gambierdiscus* spp. can be prevented in the future. Slowly dissolving silicate stone is expected to be useful for the growth enhancement of epiphytic diatoms in the macroalgae areas.

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Figure legends

Fig. 1. Location of the sampling stations in the coasts of the Sea of Japan.

Fig. 2. Seasonal changes in seawater temperature and cell densities of *Gambierdiscus* spp. attached to the red alga *Amphiroa zonata* at Niizaki, Kyoto Prefecture from March 2010 to December 2011. The bars represent cell densities of *Gambierdiscus* spp. and the solid lines represent seawater temperature.

Fig. 3. Seasonal changes in cell densities of total epiphytic diatoms and *Gambierdiscus* spp. attached to the red alga *Amphiroa zonata* at Niizaki, Kyoto Prefecture from March 2010 to December 2011. The bars represent cell densities of *Gambierdiscus spp*., the dashed lines (without symbols) represent cell densities of total epiphytic diatoms, the dashed lines (with open squares) represent cells densities of *Amphora* spp. and the dashed lines (with open triangles) represent cells densities of *Naviculoid* diatoms.

Fig. 4. Seasonal changes in cell densities of total epiphytic diatoms and dinoflagellates attached to the red alga *Amphiroa zonata* at Niizaki, Kyoto Prefecture from March 2010 to December 2011. G.spp.: cell densities of *Gambierdiscus* spp., O.spp.: *Ostreopsis* spp., P.l: *Prorocentrum lima*, P.spp.: *Prorocentrum* spp., C.spp.: *Coolia* spp., T.Di: total epiphytic diatoms.

Fig. 5. Seasonal changes of salinity and cell densities of *Gambierdiscus* spp. attached to the red alga *Amphiroa zonata* at Niizaki, Kyoto Prefecture from March 2010 to December 2011. The bars represent cell densities of *Gambierdiscus* spp. and the dashed lines (with open circles) represent salinity.

Fig. 6. Microphotographs of (A) *Gambierdiscus* sp. broad 1p type (left: Apical view, right: Antapical view, Scale bars: 20 µm) and (B) *Gambierdiscus* sp. narrow 1p type (left :Apical

view, right: Antapical view, Scale bars: 20 μm) after calcofluor staining with optical microscope.

Fig. 7. Phylogenetic tree of *Gambierdiscus* spiecies/phylotypes inferred from the D8-D10 region of the LSU rDNA. Reconstruction of the maximum likelihood (ML) trees of the D8-D10 region of the LSU rDNA from *Gambierdiscus* spiecies/phylotypes and ML bootstrap (BP) calculations (1000 replicates) were carried out using the program MEGA 5.0 (Tamura et al. 2011) with the GTR model incorporating invariable sites and a discrete gamma distribution (GTR + I + G). Sequeces obtained in the present study are represented by the bold faces.

Figure 1

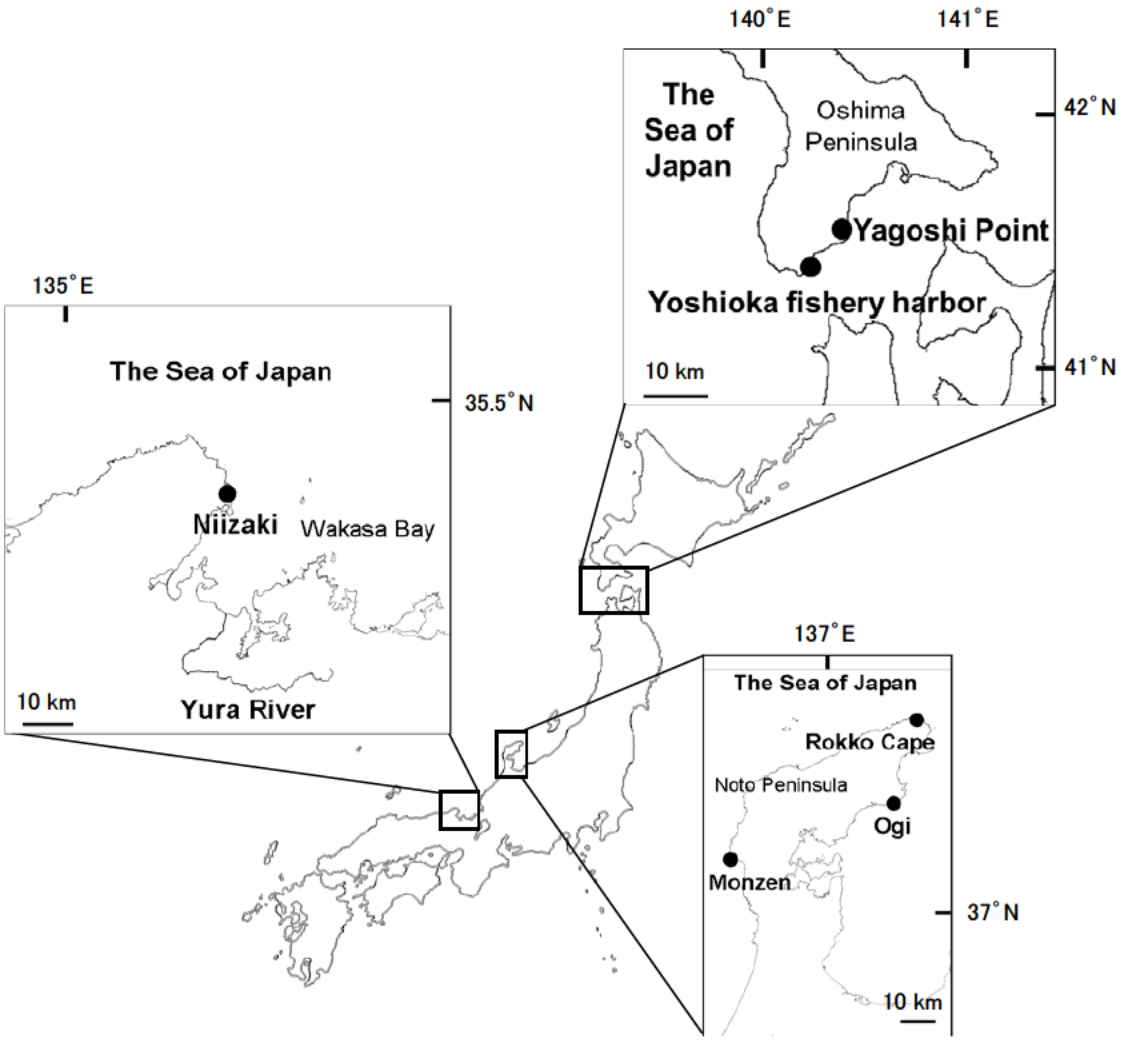

140°E
141°E
The Sea of Japan
Oshima Peninsula
42°N
Yagoshi Point
Yoshioka fishery harbor
10 km
41°N
135°E
The Sea of Japan
35.5°N
Niizaki
Wakasa Bay
10 km
Yura River
137°E
The Sea of Japan
Rokko Cape
Noto Peninsula
Ogi
Monzen
37°N
10 km

Figure 2

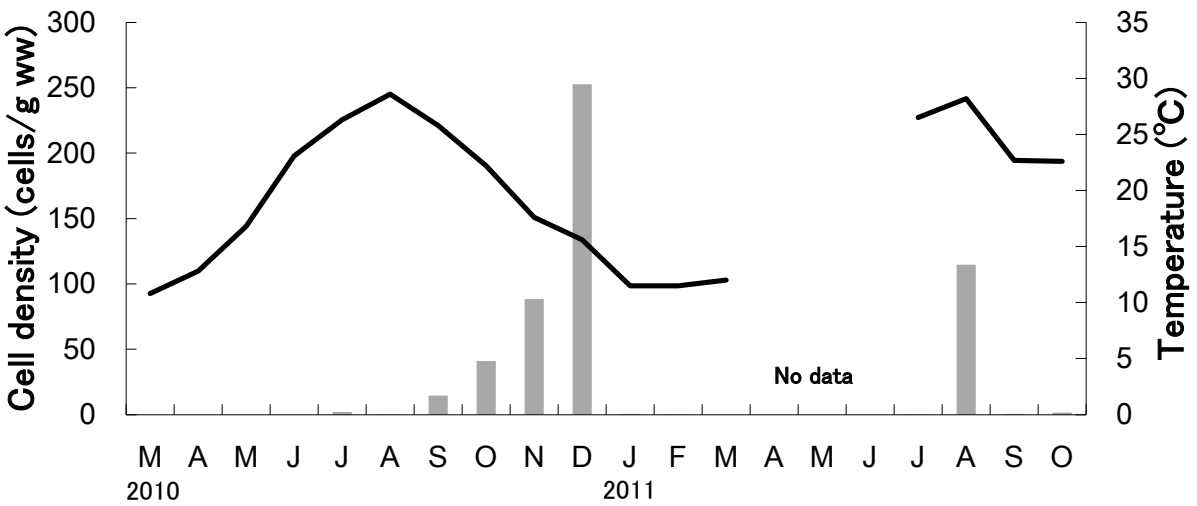

Cell density (cells/g ww)
300
250
200
150
100
50
0
35
30
25
20
15
10
5
0
Temperature (°C)
No data
M A M J J A S O N D J F M A M J J A S O
2010
2011

Figure 3

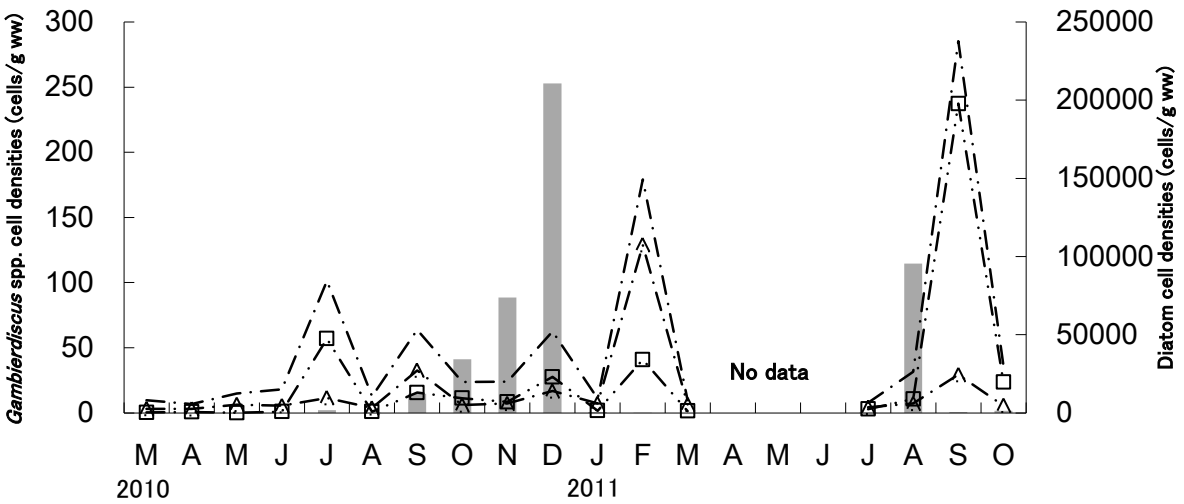

Gambierdiscus spp. cell densities (cells/g ww)
300
250
200
150
100
50
0
250000
200000
150000
100000
50000
0
Diatom cell densities (cells/g ww)
No data
M A M J J A S O N D J F M A M J J A S O
2010
2011

Figure 4

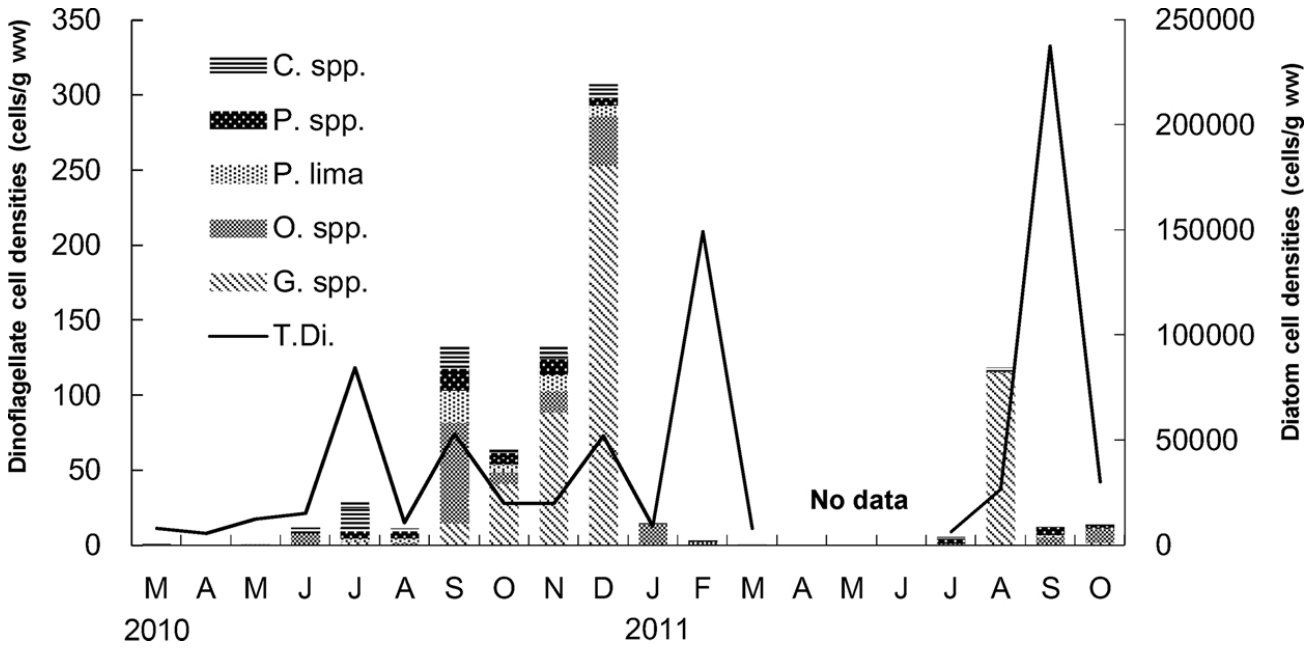

Dinoflagellate cell densities (cells/g ww)
Diatom cell densities (cells/g ww)
C. spp.
P. spp.
P. lima
O. spp.
G. spp.
T.Di.
No data
350
300
250
200
150
100
50
0
250000
200000
150000
100000
50000
0
M A M J J A S O N D J F M A M J J A S O
2010
2011

Figure 5

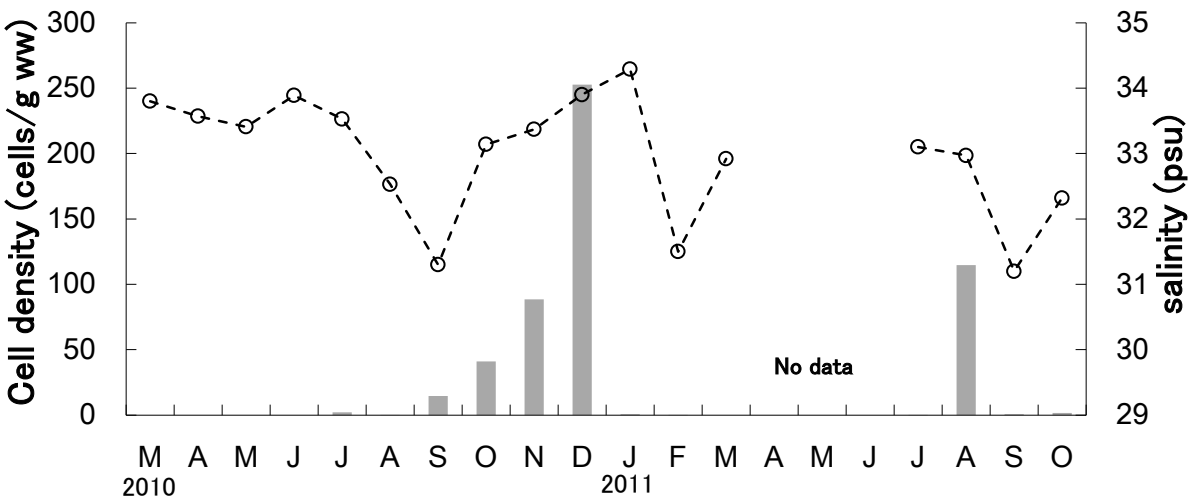

Cell density (cells/g ww)
salinity (psu)
300
250
200
150
100
50
0
35
34
33
32
31
30
29
No data
M A M J J A S O N D J F M A M J J A S O
2010
2011

Figure 6

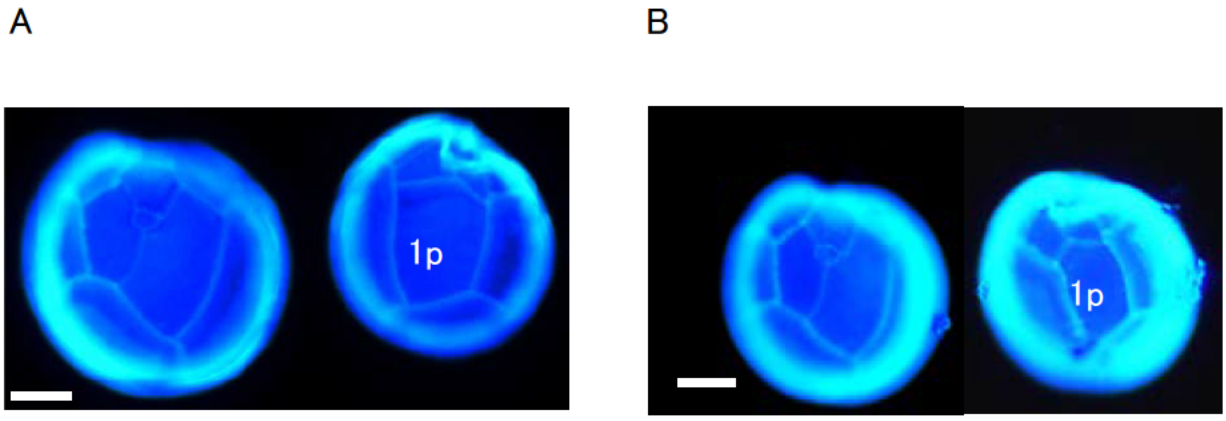
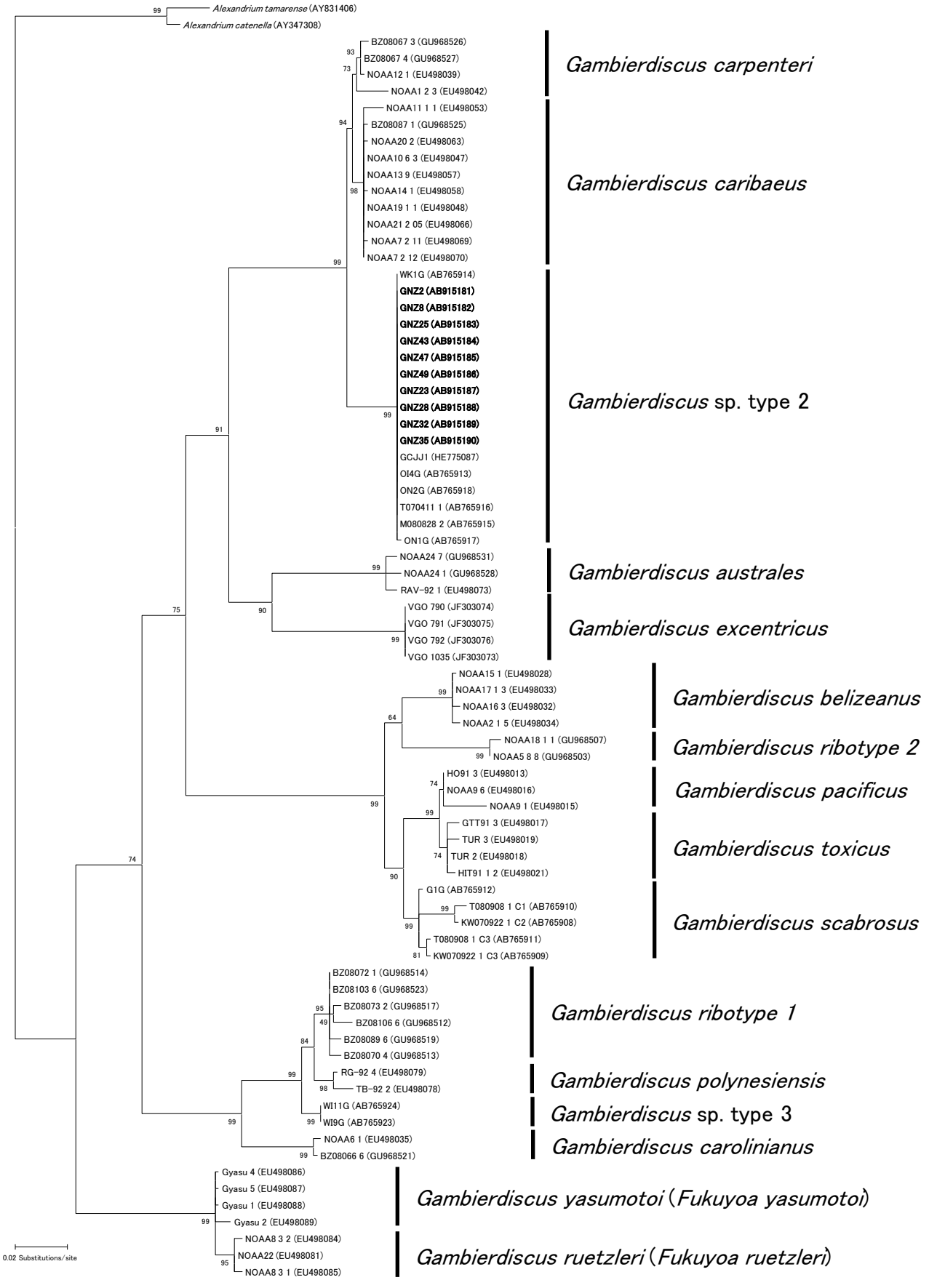


Figure 7


Alexandrium tamarense (AY831406)
Alexandrium catenella (AY347308)
BZ08067 3 (GU968526)
BZ08067 4 (GU968527)
NOAA12 1 (EU498039)
NOAA1 2 3 (EU498042)
Gambierdiscus carpenteri
NOAA11 1 1 (EU498053)
BZ08087 1 (GU968525)
NOAA20 2 (EU498063)
NOAA10 6 3 (EU498047)
NOAA13 9 (EU498057)
NOAA14 1 (EU498058)
NOAA19 1 1 (EU498048)
NOAA21 2 05 (EU498066)
NOAA7 2 11 (EU498069)
NOAA7 2 12 (EU498070)
Gambierdiscus caribaeus
WK1G (AB765914)
GNZ2 (AB915181)
GNZ8 (AB915182)
GNZ25 (AB915183)
GNZ43 (AB915184)
GNZ47 (AB915185)
GNZ49 (AB915186)
GNZ23 (AB915187)
GNZ28 (AB915188)
GNZ32 (AB915189)
GNZ35 (AB915190)
GCJJ1 (HE775087)
OI4G (AB765913)
ON2G (AB765918)
T070411 1 (AB765916)
M080828 2 (AB765915)
ON1G (AB765917)
Gambierdiscus sp. type 2
NOAA24 7 (GU968531)
NOAA24 1 (GU968528)
RAV-92 1 (EU498073)
Gambierdiscus australes
VGO 790 (JF303074)
VGO 791 (JF303075)
VGO 792 (JF303076)
VGO 1035 (JF303073)
Gambierdiscus excentricus
NOAA15 1 (EU498028)
NOAA17 1 3 (EU498033)
NOAA16 3 (EU498032)
NOAA2 1 5 (EU498034)
Gambierdiscus belizeanus
NOAA18 1 1 (GU968507)
NOAA5 8 8 (GU968503)
Gambierdiscus ribotype 2
HO91 3 (EU498013)
NOAA9 6 (EU498016)
NOAA9 1 (EU498015)
Gambierdiscus pacificus
GTT91 3 (EU498017)
TUR 3 (EU498019)
TUR 2 (EU498018)
HIT91 1 2 (EU498021)
Gambierdiscus toxicus
G1G (AB765912)
T080908 1 C1 (AB765910)
KW070922 1 C2 (AB765908)
T080908 1 C3 (AB765911)
KW070922 1 C3 (AB765909)
Gambierdiscus scabrosus
BZ08072 1 (GU968514)
BZ08103 6 (GU968523)
BZ08073 2 (GU968517)
BZ08106 6 (GU968512)
BZ08089 6 (GU968519)
BZ08070 4 (GU968513)
Gambierdiscus ribotype 1
RG-92 4 (EU498079)
TB-92 2 (EU498078)
Gambierdiscus polynesiensis
WI11G (AB765924)
WI9G (AB765923)
Gambierdiscus sp. type 3
NOAA6 1 (EU498035)
BZ08066 6 (GU968521)
Gambierdiscus carolinianus
Gyasu 4 (EU498086)
Gyasu 5 (EU498087)
Gyasu 1 (EU498088)
Gyasu 2 (EU498089)
Gambierdiscus yasumotoi (Fukuyoa yasumotoi)
NOAA8 3 2 (EU498084)
NOAA22 (EU498081)
NOAA8 3 1 (EU498085)
Gambierdiscus ruetzleri (Fukuyoa ruetzleri)
0.02 Substitutions/site