Distribution, abundance and diversity of *Gambierdiscus* spp. from a ciguatera-endemic area in Marakei, Republic of Kiribati

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Abstract

Ciguatera is a serious seafood poisoning syndrome caused by the consumption of ciguatoxin-contaminated finfish from tropical and subtropical regions. This study examined the community structure of ciguatera-associated dinoflagellates and the distribution pattern, taxonomy and toxicity of *Gambierdiscus* spp. from a high-risk area of Marakei, Republic of Kiribati. The genera *Gambierdiscus*, *Prorocentrum*, *Ostreopsis*, *Amphidinium* and *Coolia* were present, and generally the former three dominated the dinoflagellate assemblage. Among these three, *Gambierdiscus* was the most abundant dinoflagellate genus observed at three of the four sites sampled, two of which (Sites 1 and 2) were on the northern half of the island and two (Sites 3 and 4) on the southern half. The following patterns of abundance were observed among sites: (1) Average *Gambierdiscus* spp. abundance at the northern sites exceeded the southern sites by a factor of 19-54; and (2) *Gambierdiscus* spp. abundance at shallow sites (2-3 m) exceeded deeper sites (10-15 m). The distribution of *Gambierdiscus* spp. at Marakei corresponded with previously observed patterns of fish toxicity, with fish from southern locations being much less toxic than fish sampled north of the central channel. DNA sequencing identified three *Gambierdiscus* species (*G. carpenteri*, *G. belizeanus*, *G. pacificus*) and three previously unreported ribotypes (*Gambierdiscus* sp. type 4, *Gambierdiscus* sp. type 5, *Gambierdiscus* sp. type 6) in the samples; *Gambierdiscus* sp. type 4 may represent a Pacific clade of *Gambierdiscus* sp. ribotype 1. Toxicity analyses determined that *Gambierdiscus* sp. type 4 isolates were more toxic than the *Gambierdiscus* sp. type 5 and *G. pacificus* isolates, with toxin contents of 2.6-6.0 (mean: 4.3± 1.4), 0.010 and 0.011 fg

P-CTX-1 eq cell^{-1}, respectively. Despite low densities of *Gambierdiscus* spp. observed at Marakei relative to other studies in other parts of the world, the presence of low and moderately toxic populations may be sufficient to render the western coast of Marakei a high-risk area for ciguatera. The long history of toxicity along the western side of Marakei suggests that large-scale oceanographic forcings that regulate the distribution of *Gambierdiscus* spp. along the western side of Marakei may have remained relatively stable over that time. Chronic as well as acute exposure to ciguatoxins may therefore pose an important human health impact to the residents of Marakei.

Keywords: ciguatera fish poisoning; ciguatoxins; *Gambierdiscus*; *Ostreopsis*; HABs; Kiribati

1. Introduction

Ciguatera is a complex and widespread poisoning syndrome caused by the consumption of finfish that have accumulated lipid soluble toxins known as ciguatoxins. According to Lewis (2001), globally there are more than 25,000 people affected annually by ciguatera. The illness is endemic to coral reef ecosystems in tropical and subtropical areas worldwide, and particularly impacts island communities dependent on subsistence fishing. Fortunately, mortality is rare, but the gastrointestinal, neurological and cardiovascular symptoms associated with illness can be debilitating and long-lasting (Lewis, 2001).

Gambiertoxins produced by the benthic dinoflagellate genus *Gambierdiscus* are the precursors for the ciguatoxins that are ultimately responsible for ciguatera. These toxins enter the coral reef food web through grazing by herbivores and detritivores, and are accumulated and biomodified when those animals are eaten by predators. Other genera of benthic dinoflagellates produce potent toxins such as okadaic acid and pectenotoxins for *Prorocentrum* spp. (Murakami et al., 1982; Hu et al., 1995), and palytoxin for *Ostreopsis* spp. (Ramos and Vasconcelos, 2010), but have not been definitively linked to ciguatera.

Although ciguatera is distributed circumtropically, it is largely confined to islands in the Pacific Ocean, western Indian Ocean, and the Caribbean Sea (Lewis, 2001). Islands in the central Pacific arguably have more ciguatera poisoning than any other region on earth (Rongo and van Woesik, 2011). Within this region, the equatorial atoll nation of the Republic of Kiribati has a mean incidence rate almost five times as high as the Pacific region as a whole (Lewis, 1986). Ciguatera was first reported from the Line Islands Group of Kiribati in the late 1930s (Teaioro et al., 1995), and then from the Gilbert Islands in the 1940s (Cooper, 1964). Since then, ciguatera has become prevalent in Kiribati. The annual incidence rate increased from 462/100,000 in 1979-1983 (Lewis, 1986) to 1566/100,000 in 1992 (SPEHIS, 1992), though such results may only account for 20% of the actual cases that occurred (Lewis, 1986; Lewis, 2001). Given the limited land area and natural resources, Kiribati islanders heavily depend on fisheries resources for food, and also as a major revenue source. According to an overview of fisheries and aquaculture compiled by the FAO, fisheries in 2007 contributed an average of 55.8% of dietary protein, and per capita consumption of fish in Kiribati is among the highest in the world, reaching 72-207 kg/capita (FAO, 2010). The Asian Development Bank (Gillett,

2009) estimated that fishing in 2008 contributed to 8.7 % of the GDP of Kiribati; notably, a recalculation using a different methodology showed it was more than half of the official 2007 GDP of Kiribati (FAO, 2010). Therefore, the loss of fishing areas due to ciguatera greatly impacts the well-being of Kiribati islanders.

Marakei Island is the second island in the northern Gilbert Islands Group in the Republic of Kiribati. It is one of the most populated atolls in the country (Office of the President, Republic of Kiribati, 2012) with a long history of high ciguatera incidence and a comparatively detailed record of the distribution of toxic coral reef fish, which are frequently documented on the western side of the island (Cooper, 1964; Tebano and MacCarthy, 1991; Lewis, 2001; Chan et al., 2011). The first reports of ciguatera in Marakei date back to 1946, when toxic fish were caught on the northwestern side of the island near the village of Rawanawi (Cooper, 1964). The appearance of toxicity was described as sudden, and was attributed by islanders to the appearance of blue-green algae (*Schizothrix calcicola*) on certain sections of the reef. The affected area subsequently spread southward to Buota, and many more fish species gradually acquired toxicity. Surveys of *Gambierdiscus* dinoflagellate abundance carried out in Marakei in 1983 detected surprisingly low concentrations (0-4.4 cells g^{-1} algae) compared with the reported high toxicity (Tebano and MacCarthy, 1991). In this survey, the highest *Gambierdiscus* density of 4.4 cells g^{-1} algae was recorded from the reef crest near Baretoa passage (Tebano and MacCarthy, 1991). More recently, a survey of fish collected from high-risk areas on the islands of Tarawa and Marakei determined that of the 156 fish speciments collected, 91% were unsafe for consumption (Chan et al., 2011).

Thus, ciguatera remains a significant hinderance to subsistence fishing in Marakei, decades after the initial appearance of toxicity.

In an effort to help characterize the distribution, diversity, and toxicity of benthic dinoflagellate assemblages associated with ciguatera, a survey was conducted along the western coast of Marakei, Republic of Kiribati in May 2011. Results were compared with data collected previously on the prevalence of toxic fish from the sampling locations to help determine why certain locations around the island are particularly risky.

2. Materials and Methods

2.1 Study area

Marakei is a small atoll in the North Gilbert Islands of Republic of Kiribati, located in the central Pacific (2° 0' 0" N, 173° 16' 0" E). The central lagoon connects with the ocean by two narrow channels called Reweta Pass and Baretoa Pass, on the eastern and western sides of the atoll, respectively. The largest village, Rawanawi, is located on the northwestern site of the atoll. In this study, four sites were selected for sampling, all along the western side of the atoll, based on the historical incidence of toxic fish (Fig 1).

2.2 Sample collection

Field surveys were carried out from May 7-10, 2011. Samples of macroalgae and dead corals were collected at two depths (2-3 m and 10-15 m) from each of the four sites using snorkeling and/or SCUBA. For dinoflagellate enumeration, *Halimeda* sp. was the most widely distributed alga at the sampling sites (and frequently was the only algal taxa present) and was therefore selected for sampling. *Halimeda* sp. samples were cropped and sealed underwater in Ziploc bags. For sample processing, macroalgae were

vigorously shaken and kneaded for at least one minute to loosen the dinoflagellates, which were then sieved sequentially using 200 µm and 20 µm sieves. The fraction retained on the 20 µm sieve was rinsed into a 15 mL conical tube, brought up to 10 mL with filtered seawater, and preserved with 0.5 mL formalin. *Halimeda* sp. retained in the 200 µm filter was removed, blotted dry with a paper towel, and weighed. The dead coral rubble samples were placed in a bucket with filtered seawater, scrubbed with plastic brushes to remove epiphytes, and sieved as described above. Samples used for culture establishment were transferred to tissue culture flasks, and brought to 25 mL with filtered seawater and approximately 1 mL of modified K medium (Morton and Norris, 1990). Subsequent laboratory analyses were performed at the Woods Hole Oceanographic Institution, MA, USA, and the City University of Hong Kong. Samples harvested from *Halimeda* spp. were used both for enumeration and culture establishment; however, epiphytes recovered from coral rubble were only used for culture establishment.

2.3 Enumeration and culture establishment

A total of 41 samples from Sites 1-4 were enumerated, consisting of 22 samples collected from shallow locations (2-3 m depth) versus 19 samples from deeper locations (10-15 m). Samples were gently shaken and 0.5-1.0 mL was counted in a Sedgewick Rafter slide using a Zeiss Axioskop microscope at 100x magnification. Five dinoflagellate genera were enumerated: *Gambierdiscus*, *Prorocentrum*, *Ostreopsis*, *Amphidinium* and *Coolia*. Quantification results were expressed as the density of cells per gram *Halimeda* sp. (cells g^{-1} *Halimeda* sp.). In this study, only *Gambierdiscus* spp. were selected for isolation and culture establishment. Individual *Gambierdiscus* cells were isolated by micropipetting at 100x magnification, rinsed in sterile seawater three times,

and established in 25% modified K medium (Morton and Norris, 1990) and 75% sterile seawater. Isolates were subsequently transferred into tissue culture flasks and maintained in 100% modified K medium at 23°C, 32 practical salinity unit (psu), 100 $\mu mol \cdot m^{-2} s^{-1}$ of light, and 12:12h light:dark photoperiod. A total of 38 non-axenic, monoclonal cultures of *Gambierdiscus* spp. were established.

2.4 *Gambierdiscus* phylogenetic analyses

DNA was extracted from ~1mL of dense culture using the Generation Capture Column Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions, with a final elution volume of 100 µl. The LSU rRNA was amplified using primers FD8 and RB (Chinain et al., 1999). PCR reactions (25 µl) contained ~10 ng template DNA, 1 x PCR Buffer (500 mM KCL and 100 mM Tris–HCl, pH 8.3), 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 µM of each primer, and 0.5 U of AmpliTaq DNA Polymerase (Applied Biosystems Inc., Foster City, CA, USA). Hot start PCR amplifications were performed using an Applied Biosystems GeneAmp PCR system (Applied Biosystems Inc., Foster City, CA, USA) as follows: 94° C for 4 min; then 35 cycles of 94 °C for 30 s, 57 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 10 min. PCR amplification products were visualized by electrophoresis on 1% TAE agarose gel adjacent to a 100 bp DNA ladder. Positive PCR products were cloned into vector pCR 2.1 using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Clones were screened for inserts by PCR amplification with plasmid primers M13F and M13R, and eight positive clones from each PCR amplicon were selected for DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). Products were sequenced in both the forward and reverse direction.

DNA sequences were manually edited and assembled using Sequencher 4.9 (Gene Codes, Ann Arbor, MI, USA), and the consensus sequences were compared with those deposited in GenBank using BLAST sequence similarity searches (National Center for Biotechnology Information). Sequences from closely related taxa were downloaded from GenBank and aligned with the rRNA gene sequences generated by this study (Table S1). The alignment was constructed using the ClustalW (Thompson et al., 1994) and refined using MUSCLE (Edgar, 2004) as implemented in bioinformatics software Geneious Pro 6.1.2 (Biomatters, Auckland, NZ). This alignment was subsequently inspected and edited by eye. The final alignment included 149 sequences and 908 positions.

Phylogenetic trees were constructed using maximum likelihood (ML) analysis and Bayesian inference. For these analyses, Modeltest V. 3.7 (Posada and Crandall, 1998) was used to select the most appropriate model of nucleotide substitution. ML analysis was carried out using PhyML (Guindon et al., 2010), with the General Time Reversible + Gamma (GTR+G) substitution model, and 1000 bootstrap replications. Bayesian inference was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), again with the GTR+G model. Posterior probabilities were estimated using four Markov chain Monte Carlo chains, one cold and three heated, which ran for 1,000,000 generations. Trees were sampled every 400 generations following a burn-in period of 100,000 generations, after which log-likelihood values stabilized. Bayesian posterior probabilities (BPP) were calculated for each clade. For both analyses, *Gambierdiscus* sp. *ruetzleri* (GenBank Accession no. EU498083, EU498085) were used as outgroups. Genetic distances among and within *Gambierdiscus* species observed in this study and closely related taxa were also calculated. For these

calculations, consensus sequences were aligned with closely related taxa, and positions containing gaps or missing data were eliminated. Among-species distances were calculated between species/phylotypes from Kiribati, and the closest relative.

2.5 *Gambierdiscus* spp. morphological identification

In addition to the molecular identification described in 2.4, scanning electron microscopy (SEM) was used to examine the architecture of thecal plate architecture and cell surface morphology. For the SEM processing, approximately 10 mL of exponentially growing culture was preserved with glutaraldehyde (2%) and desalted with a ten step gradient from 32 psu seawater to freshwater (90%, 80%, etc., to freshwater), followed by dehydration using a ten step gradient from freshwater to 100% ethanol (10% ethanol, 20% ethanol, etc., to 100% ethanol), which was then followed by a gradient of hexamethyldisilazane (HMDS). Samples were filter-mounted to a stub and sputter coated with 1.5 nm of gold-palladium (Denton Vacuum Desk II Sputter Unit, Moorestown, NJ, USA). Measurements (length, width) of at least 20 cells observed were analyzed using MicroSuite Five (Olympus, Japan). Parameters of cell depth and width, size and shape of Apical Pore (Po), 1p, 2’ and 4’’’ were measured. For consistency and ease of comparison of our results with the scientific literature, *Gambierdiscus* were depicted by the plate tabulation nomenclature of Po, 3’, 7’’, 5’’’, 1p, 2’’’’ as described in the scientific literature (cingular and sulcus plates are not measured) (Faust, 1995; Chinain et al., 1999; Litaker et al., 2009).

2.6 *Gambierdiscus* toxicity

2.6.1 Extraction

2.1×10^6-1.1×10^7 cells from batch culture of *Gambierdiscus* in early stationary phase were harvested for toxicity detection. Ciguatoxins (CTXs) were extracted from *Gambierdiscus* cell pellets according to the procedures described by Chinain et al. (2010a) with some modifications. Cell pellets were extracted in methanol under sonication for 30 min. After centrification at 4000 rpm for 15 minutes, supernatant was collected. The extraction was repeated twice, and all the supernatant was combined. After the extract was evaporated, a solvent partition was applied to the resulting residue three times using dichloromethane and 60% aqueous methanol. The dichloromethane soluble fractions (DSFs), in which CTXs are recovered, were dried under vacuum and stored at -20^{o}C until tested for toxicity via mouse neuroblastoma assay (MNA).

2.6.2 Mouse neuroblastoma assay (MNA)

Mouse neuroblastoma (Neuro-2a cells) (ATCC, CCL131; ATCC, Manassas, VA) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Life Technologies, Carlsbad, CA) that was supplemented with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, MA), 2 g L^{-1} Na_2CO_3, antibiotic solution (50 units mL^{-1} penicillin and 50 µg mL^{-1} streptomycin) and 2.5 µg mL^{-1} Fungizone® (Gibco Life Technologies, Carlsbad, CA) at 37^{o}C in 5% CO_2. Cells were seeded at a density of 2.5×10^5 cells mL^{-1} in 96-well plate. After a 24 hour incubation, the medium was renewed with complete RPMI-1640 containing 0.1 mM ouabain and 0.01 mM veratridine. Cells were dosed with 10 uL per well extracts in three replicates. After 18 hours of incubation, cell viability was measured by MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Absorbance was measured using a microplate

reader (Molecular Devices Spectra Max 340 PC) at 595 nm with a reference wavelength of 655 nm. The optical density acquired for each well was normalized by the MTT blank.

Cells were dosed with 10 μL well^{-1} P-CTX-1 standards (purchased from Professor Lewis in University of Queensland) at seven concentrations ranging from 9.77×10^{3} fg mL^{-1} to 78.1×10^{3} fg mL^{-1} in five replicates. A standard curve of P-CTX-1 was plotted using non-linear regression ($r^2 < 0.9981$). Toxicities of *Gambierdiscus* spp. were determined based on the standard curve with a limit of quantification (LOQ) ranging from 8.7×10^{-4} to 2.1×10^{-3} fg P-CTX-1 eq cell^{-1}. Quality control of the assay was performed by testing each MNA with P-CTX-1 standard of 39.1×10^{3} fg P-CTX-1 eq. The assays were conducted twice and the toxicity values are reported as mean P-CTX-1 eq between two assays. The inter-plate relative standard deviation was 26.1%, and inter-assay relative standard deviation was 32.3%.

3. Results

3.1 Distribution and abundance of *Gambierdiscus* spp. and other ciguatera-associated dinoflagellates.

Gambierdiscus spp. were identified at all sampling sites, although patterns of abundance varied significantly (Fig 2). Generally speaking, mean abundance at the northern sites (1 and 2) exceeded the southern sites (3 and 4) by a factor of 19-54; additionally, mean abundance at shallow sites (2-3 m) exceeded the deeper sites (10-15 m) by a factor of 4-13 (Site1 and 2). Specifically, for shallow sites samples of 2-3 m, *Gambierdiscus* exhibited the following relative abundance: Site2 > Site1 > Sites 3/4, corresponding to densities of 6.7-174.8 (72.5± 61.0, n=6), 10.8-56 (26.0± 20.6, n=4), 0-1.5 (0.6± 0.6, n=5) and 0-2.2 (0.6± 0.9, n=7) cells g^{-1} *Halimeda* sp., respectively (Fig 2).

A similar pattern of abundance was observed at the deeper sites (10-15 m), where Site1 > Site2 > Sites 3/4, which corresponded with the concentration of 5.6-8.4 (7.0± 2.0, n=2), 0-20 (5.8± 6.6, n=7), 0-2.9 (1.1± 1.4, n=4), and 0-2.6 (0.8± 1.2, n=6) cells g^{-1} *Halimeda* sp., respectively (Fig 2).

In addition to *Gambierdiscus* spp., the potentially toxic dinoflagellate genera *Prorocentrum* and *Ostreopsis* were observed (Fig 3). *Prorocentrum* spp. abundances were lower than 20 cells g^{-1} *Halimeda* sp; however, *Ostreopsis* spp. varied greatly among sites, from 0-1.0 (0.3± 0.5, n=4, Site 3, 10-15 m) to 163-596 (351± 165, n=6, Site 2, 2-3 m) cells g^{-1} *Halimeda* sp. With the exception of Site 2, where *Ostreopsis* comprised the largest proportion of benthic dinoflagellates present, *Gambierdiscus* was the most abundant dinoflagellate genus observed at the four sampling sites. *Amphidinium* spp. and *Coolia* spp. co-occurred in the benthic dinoflagellate assemblage in low abundance, 0-3.5 and 0-12.9 cells g^{-1} *Halimeda* sp., respectively (data not shown in Fig 3).

3.2 *Gambierdiscus* spp. species diversity

DNA sequence data were collected from 38 *Gambierdiscus* isolates from Marakei; a subset of these isolates was selected for morphological and toxicity analyses (see below). Consensus sequences were compared with those deposited in GenBank using BLAST sequence similarity searches (National Center for Biotechnology Information), aligned with closely related taxa, and the phylogenetic relationships examined using ML and BI (Fig 4). Tree topologies of the ML and BI trees were identical, with the exception of the relationships among *G. carpenteri*, *G. caribaeus*, and *Gambierdiscus* sp. type 2. In the ML phylogeny, similar to relationships observed by Nishimura et al. (2013), *Gambierdiscus* sp. type 2 diverged first, and *G. caribaeus* and *G. carpenteri* were sister

groups. In the BI tree, however, this node was a polytomy. The relationships among described species and phylotypes were also very similar to those reported previously (Litaker et al., 2009; Fraga et al., 2011; Nishimura et al., 2013).

Examination of the phylogenetic trees confirmed the identification of three described species of *Gambierdiscus* from Kiribati: *G. carpenteri*, *G. belizeanus*, and *G. pacificus.* Additionally, we observed a clade comprised of 28 isolates, which were clustered in a well-supported sister group to the previously reported phylotype *Gambierdiscus* ribotype 1. This group comprised the largest proportion of isolates identified from Kiribati. Genetic distance values between sequences in this clade compared to sequences defined as *Gambierdiscus* ribotype 1 ranged from 0.007-0.022. The minimum number of substitutions per site (0.007) is comparable to those calculated by Fraga et al. (2011) for *G. caribaeus*/*G. carpenteri* (0.006) and *G. yasumotoi*/*G. ruetzleri* (0.008). This group may therefore represent a new phylotype/species, or potentially could comprise a Pacific clade of *Gambierdiscus* sp. ribotype 1, previously only observed in the Atlantic (Litaker et al., 2010). Until further information is available regarding the taxonomical status of this group, we have provisionally designated these sequences as *Gambierdiscus* sp. type 4, to distinguish them from previously described *Gambierdiscus* phylotypes (Litaker et al., 2009; Kuno et al., 2010; Nishimura et al., 2013).

Additionally, we identified two unique and well-supported clades, designated as *Gambierdiscus* sp. type 5 and type 6, which clustered in Clade V, as defined by Nishimura et al. (2013). Within Clade V, *G. belizeanus* and *Gambierdiscus* sp. ribotype 2 diverged first, followed by *Gambierdiscus* sp. type 1, and then *Gambierdiscus* sp. type 5. *Gambierdiscus* sp. type 6 diverged next, forming a sister group to the clade containing *G.*

toxicus and *G. pacificus*. Genetic distance values calculated for G*ambierdiscus* sp. type 5 and other closely related species/phylotypes ranged from 0.032-0.044 for the pair G*ambierdiscus* sp. type 5/*Gambierdiscus* sp. type 6, and 0.036-0.054 for the pair G*ambierdiscus* sp. type 5/ G*ambierdiscus* sp. type 1. Distances between *Gambierdiscus* sp. type 6 and closely related species ranged from 0.017-0.038 for the pair *G. pacificus*/ *Gambierdiscus* sp. type 6 and 0.024-0.047 for the pair *G. toxicus*/*Gambierdiscus* sp. type 6. DNA sequences obtained in this study were deposited into GenBank (Accession numbers: KJ125080- KJ125135).

3.4 *Gambierdiscus* morphology

The morphology of four strains from *Gambierdiscus* sp. type 4 were examined using SEM to provide a characterization of this clade, and for comparison with closely related taxa. Additionally, morphological analysis using SEM was performed on representative isolates from *Gambierdiscus* sp. type 5 (one strain), and the closely related species *G. pacificus* (one strain). Isolates of *Gambierdiscus* sp. type 6 died before the SEM work could be performed.

All isolates featured a lenticular, antero-posteriorly compressed shape covered with numerous, round, evenly scattered pores on the surface (Fig 5-7), and displayed the plate tabulation formula of Po, 3', 7'', 5''', 1p, 2''''. From the apical and antapical view, cells were round or slightly oblong. Cells of *Gambierdiscus* sp. type 4 were considerably larger and featured a smooth surface morphology compared to *Gambierdiscus* sp. type 5 and *G. pacificus* (Fig 5-7, Table 1). For *Gambierdiscus* sp. type 4, the average depth ranged from 65.9± 4.1 to 72.5± 4.2 µm and the average width from 64.5± 5.0 to 68.9± 5.0 µm. *Gambierdiscus* sp. type 5 exhibited an average depth of 54.8± 4.6 µm and

average width 53.7± 6.3 µm; for *G. pacificus*, average depth was 52.3± 3.7 µm and average width was 51.1± 4.3 µm (Table 1). *Gambierdiscus* sp. type 4 cell shape varied in depth:width ratio from 0.96-1.06. This ratio is very similar to *Gambierdiscus* sp. type 5 and *G. pacificus*, which both exhibited a depth:width ratio of 1.03 (Table 1).

The epithecae of both *Gambierdiscus* sp. types 4 and 5 and *G. pacificus* consisted of apical pore plate (Po), apical plate (') and precingular plates ('') . The Po was oval to ellipsoidal with a length-width ratio of 1.29-1.45 and the typical fishhook shaped apical pore opening (Fig 6, Table 1). Among the three apical plates, the 2' was the largest (Fig 5, Table 1). However, the epithecae morphology of *Gambierdiscus* sp. type 4 was distinguished from *Gambierdiscus* sp. type 5 and *G. pacificus* by the angle between the plate edge of 2'/1' and 2'/3', length-width ratio of 2' and ratio between the front edge of 2'/4'' and back edge of 2'/2''. For *Gambierdiscus* sp. type 4, these measurements were 86.7-94.0°, 1.38-1.46 and 1.74-1.83, whereas for *Gambierdiscus* sp. type 5 and *G. pacificus*, these measurements were 85.4°, 1.79, 1.32 and 85.4°, 1.96 1.61, respectively (Table 1). The apical plate of 2' in *Gambierdiscus* sp. type 4 was thus wide and hatchet shaped, whereas 2' in *Gambierdiscus* sp. type 5 and *G. pacificus* was long, narrow and rectangular (Fig 5, Table 1).

The hypothecae of both *Gambierdiscus* sp. types 4 and 5 and *G. pacificus* consisted of postcingular plates (''') , posterior intercalary plate (1p) and antapical plates (''''). The postcingular plates of 2''', 3''' and 4''' were large, near quadrilateral in shape (Fig 7). Distinct differences in the 1p were evident between *Gambierdiscus* sp. type 4 and members of *Gambierdiscus* type 5 and *G. pacificus*. *Gambierdiscus* sp. type 4 featured a broad 1p plate, occupying approximately 45-54% of the hypothecal width. However,

Gambierdiscus type 5 and *G. pacificus* featured a long and narrow 1p plate, apparently pentagonal, occupying approximately 25-28% of hypothecal width. For the former, 1p was wider than its 4'''', for the latter, the converse was true (Fig 7, Table 1).

3.5 *Gambierdiscus* toxicity

The same six isolates examined using SEM were also analyzed for CTX-like toxicity. All six of the strains tested produced toxins; however, differences in toxin content were observed between species (Table 2). *Gambierdiscus* sp. type 4 toxin content ranged from 2.6-6.0 fg P-CTX-1 eq cell^{-1} (4.3± 1.4 fg P-CTX-1 eq cell^{-1}, n=4); toxicity of *Gambierdiscus* sp. type 5 and *G. pacificus* were very similar, corresponding to 0.010 fg P-CTX-1 eq cell^{-1} (n=1) and 0.011 (n=1) fg P-CTX-1 eq cell^{-1}, respectively. *Gambierdiscus* sp. type 4 in Marakei was up to two orders of magnitude more toxic than *Gambierdiscus* sp. type 5 and *G. pacificus*. In contrast to interspecific toxicity differences, intraspecific toxicity of *Gambierdiscus* sp. type 4 fluctuated little (Table 2).

4. Discussion

This study documented several ciguatera associated benthic dinoflagellate genera from Marakei, Kiribati, including *Gambierdiscus*, *Prorocentrum*, *Ostreopsis*, *Amphidinium* and *Coolia*. The former three predominated in abundance in assemblages; among these three, *Gambierdiscus* was generally most abundant. Differences were observed among sites: *Gambierdiscus* abundance was higher in the north than in the south of the island, and at shallow sites versus deep; the former pattern was consistent with historical fish toxicity distribution patterns on the western side of the island. Morphological and phylogenetic analyses identified three species and three previously undescribed ribotypes. Toxicity analysis of six isolates comprising one species and two

ribotypes showed that all produced toxins, but one ribotype was far more toxic than the others. When our study data are combined with descriptions of the distribution of *Gambierdiscus* and toxic fishes over several decades, it appears that oceanographic forcings that regulate the distribution of *Gambierdiscus* spp. around the island may have remained relatively stable over time. These and other issues are discussed in more detail below.

4.1 History of ciguatera in Marakei

For many of the Gilbert Islands comprising the Republic of Kiribati, ciguatera first became a problem for affected islands in the 1940s, and was blamed on reef damage caused by construction, the dumping of rubbish, increased shipping, and bombs (Cooper, 1964). On Marakei, ciguatera appeared suddenly in 1946, when toxic fishes were first caught from reefs near the Rawanawi village (near Site1). Toxicity then extended southwards to Buota village (Cooper, 1964) and subsequently expanded further southward from Buota village. By 1983, toxic areas included almost the entire western reef from Rawanawi (near Site1) to Tekarakan villages (near Site2) (Tebano and MacCarthy, 1991). In contrast with other islands in Kiribati, in which increases in ciguatera were linked to anthropogenic activities, islanders on Marakei related the sudden appearance of ciguatera to a filamentous blue-green alga *Schizothrix calcicola* (cyanobacteria) that previously was not observed. This mat-forming alga grew on top of existing substrate and first appeared in the vicinity of Rawanawi, then spread to Buota (Cooper, 1964). Although anecdotal, this observation is interesting given subsequent work reporting that the cyanobacterium *Oscillatoria erythraea* can produce CTX-like toxins (Hahn and Capra, 1992). More recently, the cyanobacterium *Hydrocoleum*

Kützing was related to CFP-like outbreaks following human consumption of giant clams on Lifou Island, New Caledonia (Laurent et al., 2008). Similar incidents were reported in French Polynesia and in the Republic of Vanuatu in the Pacific Ocean (Laurent et al., 2012), where CTX-like and paralyzing toxins were confirmed both in cyanobacteria and the molluscs. The authors thus proposed to use "Ciguatera shellfish poisoning (CSP)" instead of the traditional "Ciguatera fish poisoning (CFP)" to describe this new ciguatera-related ecotoxicological phenomenon (Laurent et al., 2012). We did not investigate the occurrence or toxicity of cyanobacteria in the current study; however, the current study found biofilm, instead of macroalgae, was in the diet of herbivorous surgeonfish and parrotfish (L. Chan, personal communication). Mat-forming cyanobacteria such as *Schizothrix* sp. might serve as a preferred host for *Gambierdiscus* spp. at Marakei, thus harboring high cell densities, and/or may also be a source of CTX-like compounds. Examining the potential link between ciguatoxicity and cyanobacteria on Marakei in the future may be informative for understanding the initial appearance and persistence of toxicity on the island.

From 1978-1990, annual cases of fish poisoning varied from 3-369 in Republic of Kiribati excluding Tarawa; 11%-52% of the cases were from Marakei alone (calculated from Tebano and MacCarthy, 1991). This same study identified *Gambierdiscus* spp. near field sites sampled during the current study (Tebano and MacCarthy, 1991; MRAM, 1999) at densities ranging from 0-4.4 cells g^{-1} macroalgae (mixed assemblage), which are low compared to other reports from Pacific islands, e.g., 0-4,871 cells g^{-1} wet weight macroalgae in Jeju Island, Korea (Kim et al., 2011) and 0-141,890 cells g^{-1} wet weight host algae in French Polynesia (Chinain et al., 2010b). These low cell densities were in

contrast to the reported high toxicity of fishes collected from these locations (Tebano and MacCarthy, 1991). Recently, Chan et al. (2011) reported toxin levels in excess of 1 ng g^{-1} P-CTX-1 equivalents in fish from the northwestern side of Marakei (near Sites 1 & 2) but not from locations along the southwestern coast (near Sites 3 & 4). Surveys conducted during the current study again showed patterns in which toxin levels in fish from Sites 3 and 4 were 85-1240 fold lower than those from Sites 1 and 2 (L. Chan, personal communication). Detailed information on seasonal or annual fluctuations in fish toxicity is unavailable, but the consistent view that emerges from studies that span over three decades is that fish from the northern part of the island are significantly more toxic than those from the south.

4.2 Dinoflagellate distribution

Gambierdiscus spp. abundance varied from 0-175 cells g^{-1} *Halimeda* sp. among sites sampled at Marakei. These densities are higher than densities recorded on *Halimeda* sp. as well as other algal taxa during previous surveys at Marakei (Tebano and MacCarthy, 1991; MRAM, 1999), but are much lower than cell densities observed on *Halimeda* sp. collected from other regions of the world (Table 3). For example, these levels correspond to approximately 4% and 17% of the highest abundances in Knight Key, Florida and French Polynesia, respectively (Table 3). However, the limited scope and duration of sampling in the current study (comprising a single survey), as well as those carried out previously, fails to capture seasonal fluctuations in *Gambierdiscus* spp. abundance; temporal sampling is thus needed to determine the true range of cell densities at Marakei.

In general, dinoflagellate abundance was much higher at the two northern sites compared with the southern sites, a pattern that corresponds well to historical patterns of

fish toxicity. The prevalence of toxic fish on the western side of Marakei is also similar to other islands in the Gilbert Islands group, in which toxicity is most prevalent in habitats on the sheltered, lee side (Cooper, 1964). Sheltered habitats such as inshore island, inner slope of barrier reef, and lagoon have been shown to support high *Gambierdiscus* abundances; conversely habitats experiencing strong wind and water motion generally sustain lower abundances (Carlson and Tindall, 1985; Grzebyk et al., 1994; Tindall and Morton, 1998; Faust, 2009; Richlen and Lobel, 2011). Throughout the Gilbert Islands Group, the prevailing wind comes from northeast, east or southeast, and the current is from the southeast (Cooper, 1964); Sites 1 and 2 are therefore less influenced by trade winds and currents than Site 3 and 4 (Fig 1), which may contribute the persistence of *Gambierdiscus* dinoflagellates and ciguatera toxicity at the former two locations.

In addition to site differences, we also observed differences among the sampling depths, with shallower sites generally supporting higher dinoflagellate abundance. This agrees with Carlson and Tindall (1985), who demonstrated benthic dinoflagellate diversity and abundance was greatest in 0.5-3.0 m. However, there is no general consensus concerning the preferences and tolerances of *Gambierdiscus* spp. to irradiance levels. Culture-based experiments have indicated that strong irradiance deterred growth, and that *Gambierdiscus* spp. adapted to relatively low light levels, e.g., 10% of full sunlight (Yasumoto et al., 1980; Bomber et al., 1989; Morton et al., 1992; Kibler et al., 2012). Conversely, *Gambierdiscus* can survive and spread using drift algae as a host, which may be indicative of a high tolerance to light intensity (Bomber et al., 1988b). Light intensities were not measured at the sampling sites at Marakei, so the role of

irradiance versus other factors in determining abundance differences between the two sampling depths is unclear.

4.3 *Gambierdiscus* diversity

Thus far, 11 species and 5 ribotypes of *Gambierdiscus* have been described. Among them, *G. carpenteri* and *G. caribaeus* are widespread (Litaker et al., 2010). Members of *G. excentricus* and *Gambierdiscus* sp. types 1, 2, and 3 are so far only recorded in the Canary Islands in the NE Atlantic Ocean and the southern part of Japan in the Pacific, respectively (Kuno et al., 2010; Fraga et al., 2011; Nishimura et al., 2013). *G. belizeanus* was previously regarded as a Caribbean species (Litaker et al., 2010) but was recently identified in Malaysia and in the Gulf of Aqaba in Jordan (Leaw et al., 2011; Saburova et al., 2013); similarly, *G. yasumotoi* was previously reported only from the Pacific (Litaker et al., 2010), but was was recently found in southern Kuwait coast and in the Gulf of Aqaba in Jordan (Saburova et al., 2013). Thus, these two species may be globally distributed as well. Despite the spatial and temporal limitations to our sampling, we identified three *Gambierdiscus* spp. and three ribotypes from Marakei in the central Pacific: *G. carpenteri, G. pacificus, G. belizeanus* and *Gambierdiscus* sp. types 4, 5, and 6 from four days sampling. Compared with the morphology of *G. pacificus* collected from Tuamotu Archipelago, Pacific Ocean (Chinain et al., 1999; Litaker et al., 2009), *G. pacificus* cells from Marakei are rounder with D:W 1.03 versus 1.11, the Po is longer, with L:W 1.39 versus 1.27, and the 1p plate occupies more space in the hypotheca with 28% versus 20%. This morphological variability may reflect intraspecific geographical distinction, also observed in other *Gambierdiscus* species (Litaker et al., 2009). *Gambierdiscus* sp. type 4 isolates from Marakei are morphologically similar to the

phylogenetically close group of *G. polynesiensis* in cell size, with average depth 65.9-72.5 µm versus 69-70 µm, average width 64.5-68.9 µm versus 69-71 µm, and similar 1p width:hypothecal width ratio of 45-54% versus 45-46% (Chinain et al., 1999; Litaker et al., 2009). *Gambierdiscus* sp. type 5 is very close to *G. pacificus* from Marakei (Table 3) but smaller than *G. pacificus* isolated from Tuamotu Archipelago, Pacific Ocean (Chinain et al., 1999; Litaker et al., 2009). In addition, the *Gambierdiscus* sp. type 5 isolate is obviously smaller than the closely related species *G. toxicus*, e.g., cell depth 54.8 µm versus 93.0 µm (Chinain et al., 1999; Litaker et al., 2009).

This study represents the first documented occurrence of *G. belizeanus* in Micronesia, and the description of *Gambierdiscus* sp. types 4, 5, and 6 in the Pacific. *Gambierdiscus* sp. type 4 is closely related to *Gambierdiscus* sp. ribotype 1, and may represent a Pacific clade of this species, which previously was only observed in the Atlantic. There are no published data on the morphology of *Gambierdiscus* sp. ribotype 1 available yet, so morphological comparisons cannot be carried out between these two phylotypes. As more field investigations of *Gambierdiscus* spp. include characterizations of species diversity, the known distribution of species similarly described as 'geographically restricted' may eventually expand.

4.4 Toxicity of *Gambierdiscus* spp. from Marakei

Neither *Gambierdiscus* sp. type 4 and type 5 nor *G. pacificus* were highly toxic, with CTX toxin contents of 4.3± 1.4, 0.01 and 0.01 fg P-CTX-1 eq cell^{-1}, respectively. The toxicity of *G. pacificus* in the present study is similar to that of conspecific strains from French Polynesia (Chinain et al., 2010a; Pawlowiez et al., 2013), but significantly lower than one isolate from Malaysia, which ranged from 31.7-75.8 fg P-CTX-1 eq cell^{-1}

(Caillaud et al., 2011). Given the relatively low *Gambierdiscus* densities (Table 3) and low toxicity of the isolates examined, the prevalence of ciguatera in Marakei is surprising, that is, 91% of fish sampled previously exceeded the 0.01 ng g^{-1} PCTX-1 eq quarantine threshold (Chan et al., 2011). One possible explanation is that since *G. carpenteri*, *G. belizeanus* and *Gambierdiscus* sp. type 6 from Marakei are known to be ciguatoxin producers, they also contribute toxins to the local food web. We are unable to test this hypothesis due to the lack of cultures, but *G. belizeanus* analyzed by Chinain et al. (2010a) produced CTX levels of 15.4 fg P-CTX-1 eq $cell^{-1}$, which is 3.6-fold more toxic than *Gambierdiscus* sp. type 4 from Marakei.

An alternative explanation may relate to the host alga selected for sampling, *Halimeda* sp., which may not support high *Gambierdiscus* spp. cell densities compared with more palatable algal taxa consumed by reef fish, and because of its calcareous structure. Cell abundances normalized to a gram of algal host may give low values relative to the same number of cells normalized to the mass of a different host. A third explanation is that the stable presence of low and moderately toxic populations of *Gambierdiscus* spp. is indeed sufficient to render the western coast of Marakei a high-risk area for ciguatera, given that the toxins bioaccumulate through time. A final possible explanation is the potential contribution of other toxic algal species was not detected during the present study, due to its limited sampling duration and scope. The potential for toxicity from cyanobacteria (Hahn and Capra, 1992; Laurent et al., 2008; Laurent et al., 2012) cannot be ignored in this regard. One way or the other, the long history of toxicity at northern versus southern regions on the west side of the island suggests that the oceanographic drivers of *Gambierdiscus* species composition and abundance (and thus

toxicity) have remained relatively stable over the last several decades. This further suggests that in addition to outbreaks that cause sudden and high toxicity, chronic exposure to ciguatoxins may be an important human heath impact to the residents of Marakei.

5. Conclusion

Marakei is an atoll that has struggled for many years with the impacts of ciguatera on human health and subsistence fishing. As a follow-up to surveys documenting the distribution of toxic fish around the atoll, we characterized the distribution and diversity of *Gambierdiscus* spp. populations using microscopy and DNA sequencing, and measured toxin content using MNA. *Gambierdiscus* and other potentially toxic dinoflagellate genera (*Prorocentrum, Ostreopsis, Amphidinium* and *Coolia*), previously considered as associated or potentially associated with ciguatera, were documented at four sampling sites on the western side of the atoll. *Gambierdiscus* spp. populations on Marakei were characterized by a high level of species diversity: three species and three previously unreported ribotypes of *Gambierdiscus* were identified in the samples, one of which may represent a Pacific clade of *Gambierdiscus* sp. ribotype 1. This study also provides the first report of *G. belizeanus* from Micronesia. The distribution of *Gambierdiscus* spp. at Marakei corresponded with previously observed patterns of fish toxicity, with fish from southern locations being much less toxic than fish sampled north of the central channel. Despite the limited dataset, this field study indicates *Gambierdiscus* spp. populations in Marakei are characterized by high biodiversity, and that cell densities provide a first order indication of the potential for toxicity at each

locale. Further studies are needed to determine whether additional, perhaps highly toxic *Gambierdiscus* spp. are present, and if not, how fish can reach dangerous levels of toxicity in areas that may only support low and moderately toxic species and strains of *Gambierdiscus*. Additional studies are also needed on how the environmental characteristics of reef ecosystems at Marakei, including how the presence and toxicity of cyanobacteria have contributed to the apparent persistence of toxicity over the last several decades.

Acknowledgements:

The authors thank the Ministry of Fisheries & Marine Resources Development (MFMRD) of the Government of Kiribati for their support, the residents of Marakei for their kindness, hospitality and assistance with field activities, and the China Scholarship Council for their financial support. We also thank Taylor Sehein for her assistance and two anonymous reviewers for their comments. Funding for this work was provided by the Centers for Disease Control and Prevention (U01 EH000421), USFDA (F223201000060C), NOAA NOS (Cooperative Agreement NA11NOS4780060, NA11NOS4780028), National Program on Key Basic Research Project of China (973 Program, 2013CB956503), the Nonprofit Research Project for the State Oceanic Administration (China, 201005006-01), and the National Natural Science Foundation of China (41276110). This is ECOHAB contribution number ECO787.

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Table 1. Cell size measurements for *Gambierdiscus* sp. type 4, *Gambierdiscus* sp. type 5 and *G. pacificus* collected from Marakei: cell depth and width, apical pore plate Po, apical plate 2', posterior intercalary plate 1p, and postcingular plate 4'''.

		Cell			Apical Pore			2'							1p			4'''		
Sample	Species Name	D	W	D:W	L	W	L:W	Angle	L	W	L:W	Front	Back	F:B	L	W	L:W	L	W	L:W
		(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)	(s)
1S00-04	*Gambierdiscus* sp.	65.9	68.9	0.96	6.9	5.1	1.35	90.3	33.4	24.3	1.38	20.7	11.7	1.79	39.7	31.2	1.28	56.1	18.3	3.22
	type 4	(4.1)	(5.5)	(0.05)	(0.5)	(0.5)	(0.14)	(11.4)	(2.6)	(2.3)	(0.12)	(2.3)	(1.8)	(0.29)	(4.5)	(3.5)	(0.19)	(4.4)	(4.6)	(0.71)
1D00-01	*Gambierdiscus.*	67.0	68.8	0.97	7.1	5.5	1.29	91.7	37.0	25.7	1.46	22.7	13.1	1.79	40.0	33.0	1.23	52.2	15.9	3.36
	sp. type 4	(5.2)	(5.6)	(0.06)	(0.7)	(0.7)	(0.19)	(10.1)	(3.0)	(3.0)	(0.20)	(2.9)	(2.2)	(0.39)	(4.1)	(5.2)	(0.15)	(5.7)	(2.9)	(0.58)
1D0509-16	*Gambierdiscus.*	72.5	68.6	1.06	7.3	5.1	1.45	86.7	37.1	27.1	1.40	22.9	13.5	1.74	43.3	33.5	1.30	53.7	17.7	3.26
	sp. type 4	(4.2)	(5.8)	(0.10)	(0.9)	(0.5)	(0.25)	(10.4)	(2.8)	(3.5)	(0.31)	(3.0)	(2.4)	(0.33)	(4.6)	(2.7)	(0.14)	(7.7)	(4.6)	(1.06)
1D0510-22	*Gambierdiscus.*	67.1	64.5	1.04	7.2	5.5	1.33	94.0	36.3	25.6	1.43	22.1	12.5	1.83	43.3	35.0	1.26	55.6	14.0	4.07
	sp. type 4	(4.2)	(5.0)	(0.06)	(0.5)	(0.7)	(0.18)	(5.8)	(3.1)	(2.8)	(0.19)	(3.0)	(2.4)	(0.44)	(5.1)	(5.4)	(0.23)	(6.0)	(2.6)	(0.61)
DS0511-03	*Gambierdiscus.*	54.8	53.7	1.03	5.0	3.6	1.39	86.4	27.8	15.6	1.79	13.0	10.0	1.32	26.6	13.5	2.01	42.6	24.7	1.80
	sp. type 5	(4.6)	(6.3)	(0.09)	(0.3)	(0.4)	(0.18)	(7.8)	(2.4)	(1.6)	(0.20)	(1.2)	(1.0)	(0.17)	(2.2)	(1.8)	(0.34)	(4.1)	(4.9)	(0.44)
3S0509-27	*G. pacificus*	52.3	51.1	1.03	5.6	4.1	1.39	85.4	27.0	14.1	1.96	12.9	8.1	1.61	27.3	14.5	1.91	41.0	22.5	1.85
		(3.7)	(4.3)	(0.07)	(0.4)	(0.4)	(0.14)	(11.6)	(2.0)	(1.9)	(0.38)	(1.6)	(1.1)	(0.17)	(1.7)	(1.7)	(0.25)	(4.1)	(3.6)	(0.24)

Values represent the mean measurement of examined specimens, (s) indicates standard deviation, n=20.
Among these indices, cell depth (D) refers to ventral-dorsal distance, cell width (W) refers to transdiameter across cell depth, 2' Front and 2' Back represent 2'/4'' edge and 2/2'' edge, respectively.

Table 2. *Gambierdiscus* toxicity in Marakei, Republic of Kiribati.

Sample	Species Name	Toxicity (fg P-CTX-1 eq cell^{-1})
1S00-04	*Gambierdiscus* sp. type 4	4.4
1D00-01	*Gambierdiscus* sp. type 4	2.6
1D0509-16	*Gambierdiscus* sp. type 4	4.1
1D0510-22	*Gambierdiscus* sp. type 4	6.0
DS0511-03	*Gambierdiscus* sp. type 5	0.010
3S0509-27	*G. pacificus*	0.011

Table 3. *Gambierdiscus* abundance comparison among regions (cells g^{-1} *Halimeda* sp.).

Regions	*Gambierdiscus* (cells g^{-1} *Halimeda* sp.)	References
O'ahu, Hawai'i	0	McCaffrey et al. (1992)
Great Barrier Reef, Australia	0	Heil et al. (1998)
Fiji Islands, Pacific	0- 0.01	Inoue and Raj (1985)
Queensland, Australia	7	Gillespie et al. (1985)
North Line Island, central Pacific	0-10	Briggs and Leff (2007)
French Polynesia	34	Yasumoto et al. (1979)
Marakei, central Pacific	0- 174	This study
Barrier mangrove, Belize	35- 300	Faust (2009)
Belizean Barrier Reef	0- 420	Morton and Faust (1997)
Florida Keys	336- 647	Bomber et al. (1989)
Raivavae Island, French Polynesia	0- 1023	Chinain et al. (2010b)
Knight Key, Florida	3- 4774	Bomber et al. (1988a)

Figure legends

Figure 1. Sampling map of Marakei, Republic of Kiribati.

Figure 2. *Gambierdiscus* spp. abundance at 2-3 m and 10-15 m from site 1-4 in Marakei, Republic of Kiribati. Data is expressed as average± SD.

Figure 3. Abundance of ciguatera-associated dinoflagellates at 2-3 m (Figure 3A) and 10-15m (Figure 3B) observed at site 1-4 in Marakei, Republic of Kiribati. Data is expressed as average± SD. Total dinoflagellates abundance includes the genera *Gambierdiscus*, *Prorocentrum*, *Ostreopsis, Amphidinium and Coolia*. Note differences in y-axis scale between Figure 3A and 3B.

Figure 4. Bayesian inference (BI) phylogeny generated using the D8-D10 region of the LSU rRNA gene of DNA of *Gambierdiscus* species/phylotypes. Scale bar = 0.05 substitutions per site. Supports at internal nodes are Bayesian posterior probability (pp) and bootstrap support values (>60%) from maximum likelihood (ML) analysis. Symbol of *** indicates isolates used in SEM and toxicity analyses.

Figure 5. Scanning electron micrograph images of apical view of *Gambierdiscus* sp. type 4: (A) 1S00-04; (B) 1D00-01; (C) 1D0509-16; (D) 1D0510-22; *Gambierdiscus* sp. type 5: (E) DS0511-03; and *G. pacificus*: (F) 3S0509-27. Scale bar: 10 µm.

Figure 6. Scanning electron micrograph images of apical pore of *Gambierdiscus* sp. type 4: (A) 1S00-04; (B) 1D00-01; (C) 1D0509-16; (D) 1D0510-22; *Gambierdiscus* sp. type 5: (E) DS0511-03; and *G. pacificus*: (F) 3S0509-27. Scale bar: 2.5 µm.

Figure 7. Scanning electron micrograph images of antapical view of *Gambierdiscus* sp. type 4: (A) 1S00-04; (B) 1D00-01; (C) 1D0509-16; (D) 1D0510-22; *Gambierdiscus* sp. type 5: (E) DS0511-03; and *G. pacificus*: (F) 3S0509-27. Scale bar: 10 µm.

Figure 1.

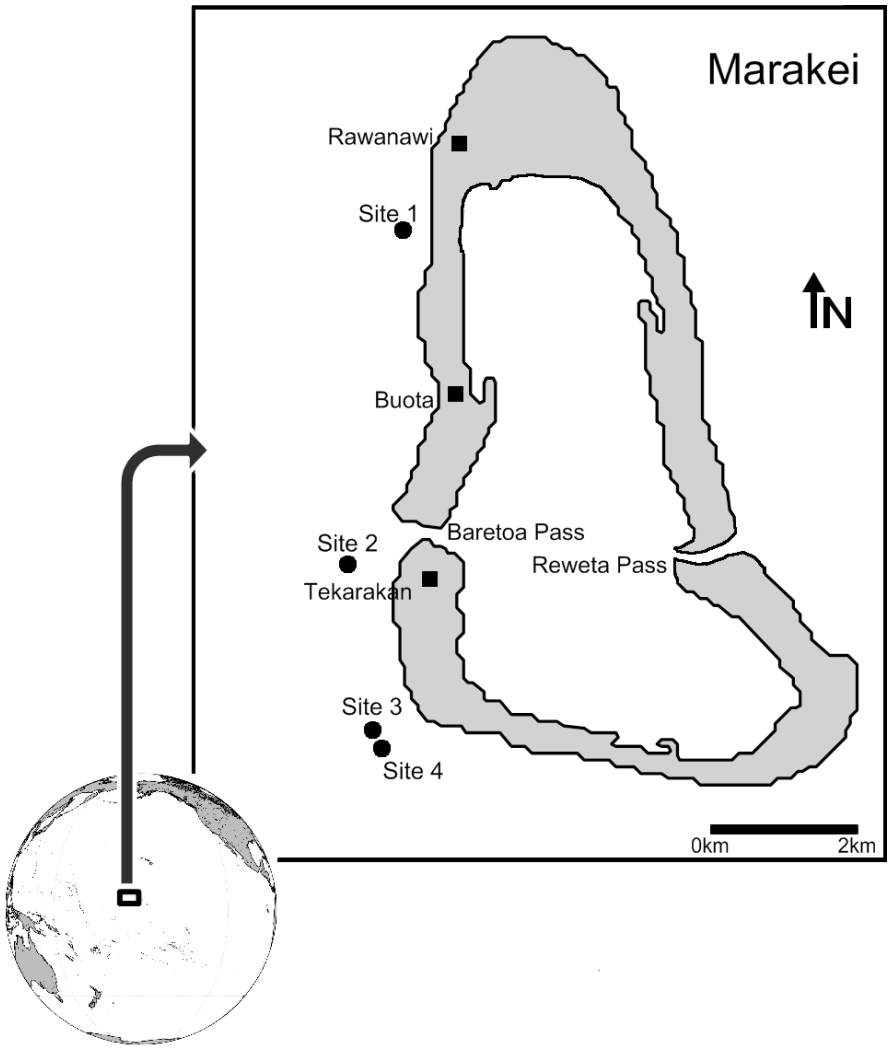

Marakei
Rawanawi
Site 1
N
Buota
Baretoa Pass
Site 2
Reweta Pass
Tekarakan
Site 3
Site 4
0km
2km

Figure 2.

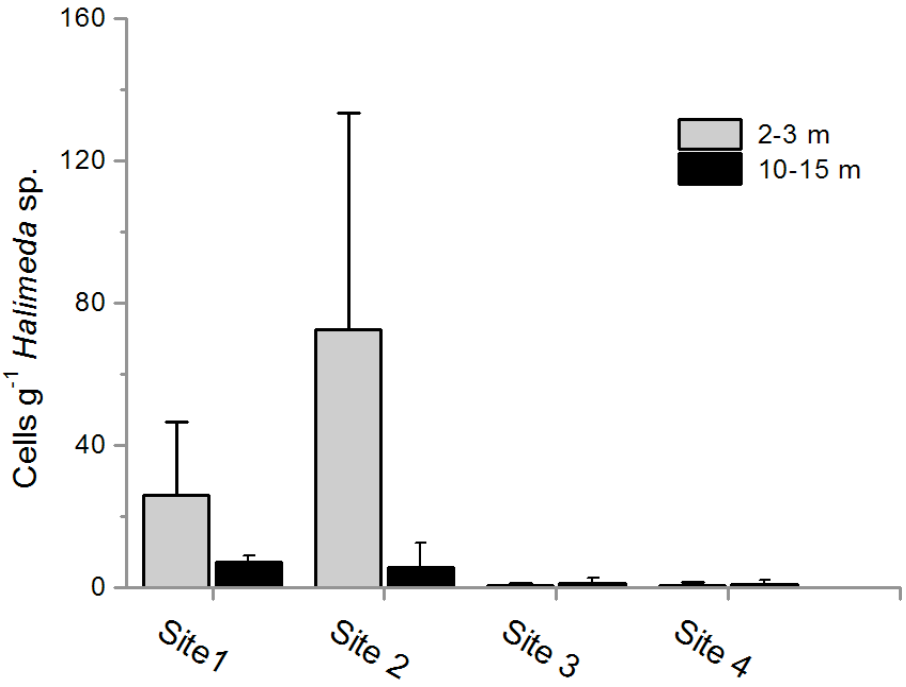

160
120
80
40
0
Cells g-1 Halimeda sp.
2-3 m
10-15 m
Site 1
Site 2
Site 3
Site 4

Figure 3.

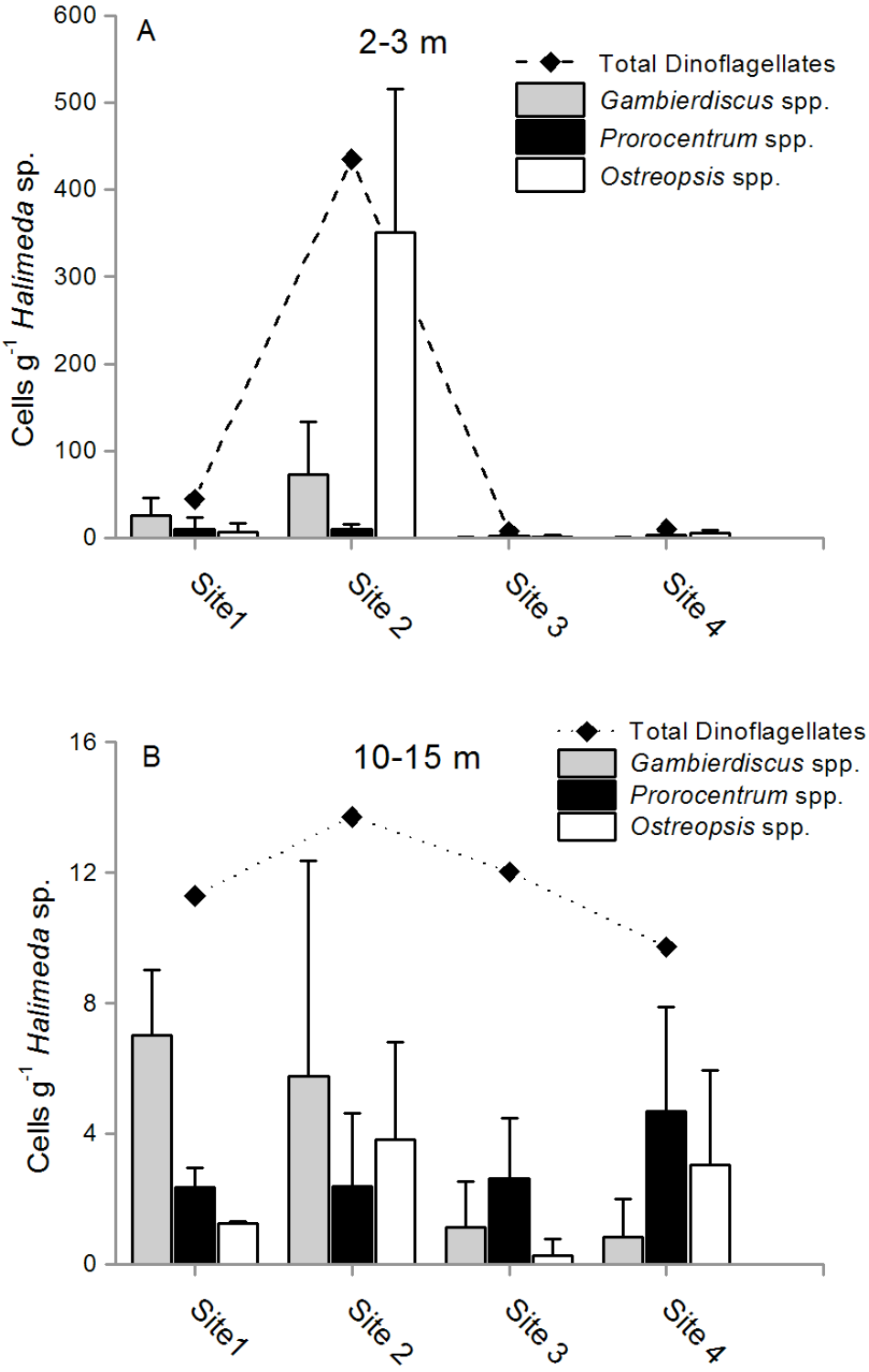

A
2-3 m
Total Dinoflagellates
Gambierdiscus spp.
Prorocentrum spp.
Ostreopsis spp.
Cells g-1 Halimeda sp.
600
500
400
300
200
100
0
Site1
Site 2
Site 3
Site 4
B
10-15 m
Total Dinoflagellates
Gambierdiscus spp.
Prorocentrum spp.
Ostreopsis spp.
Cells g-1 Halimeda sp.
16
12
8
4
0
Site1
Site 2
Site 3
Site 4

Figure 4.

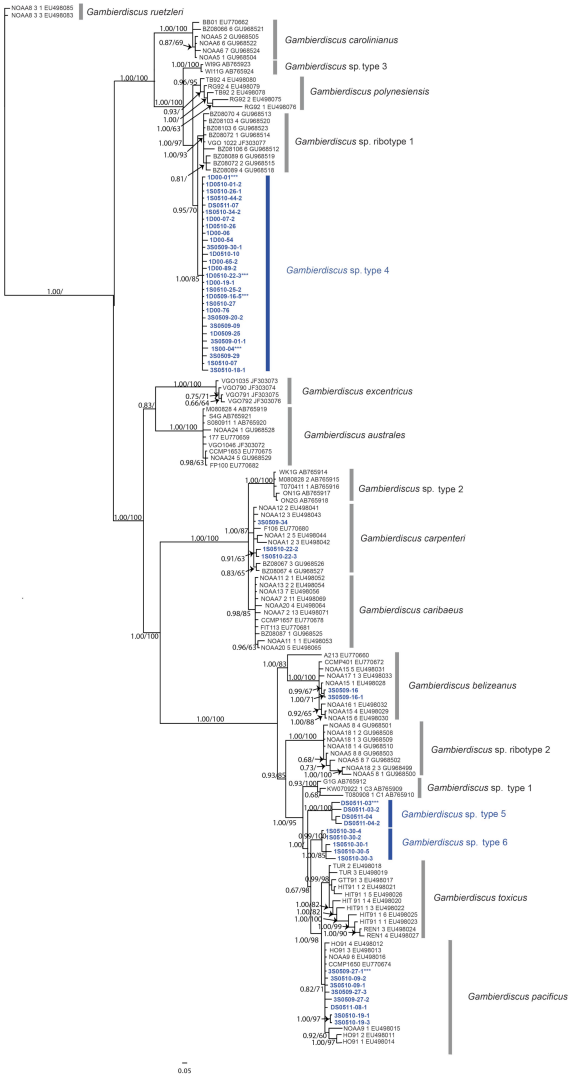

Gambierdiscus ruetzleri
Gambierdiscus carolinianus
Gambierdiscus sp. type 3
Gambierdiscus polynesiensis
Gambierdiscus sp. ribotype 1
Gambierdiscus sp. type 4
Gambierdiscus excentricus
Gambierdiscus australes
Gambierdiscus sp. type 2
Gambierdiscus carpenteri
Gambierdiscus caribaeus
Gambierdiscus belizeanus
Gambierdiscus sp. ribotype 2
Gambierdiscus sp. type 1
Gambierdiscus sp. type 5
Gambierdiscus sp. type 6
Gambierdiscus toxicus
Gambierdiscus pacificus
0.05

Figure 5.

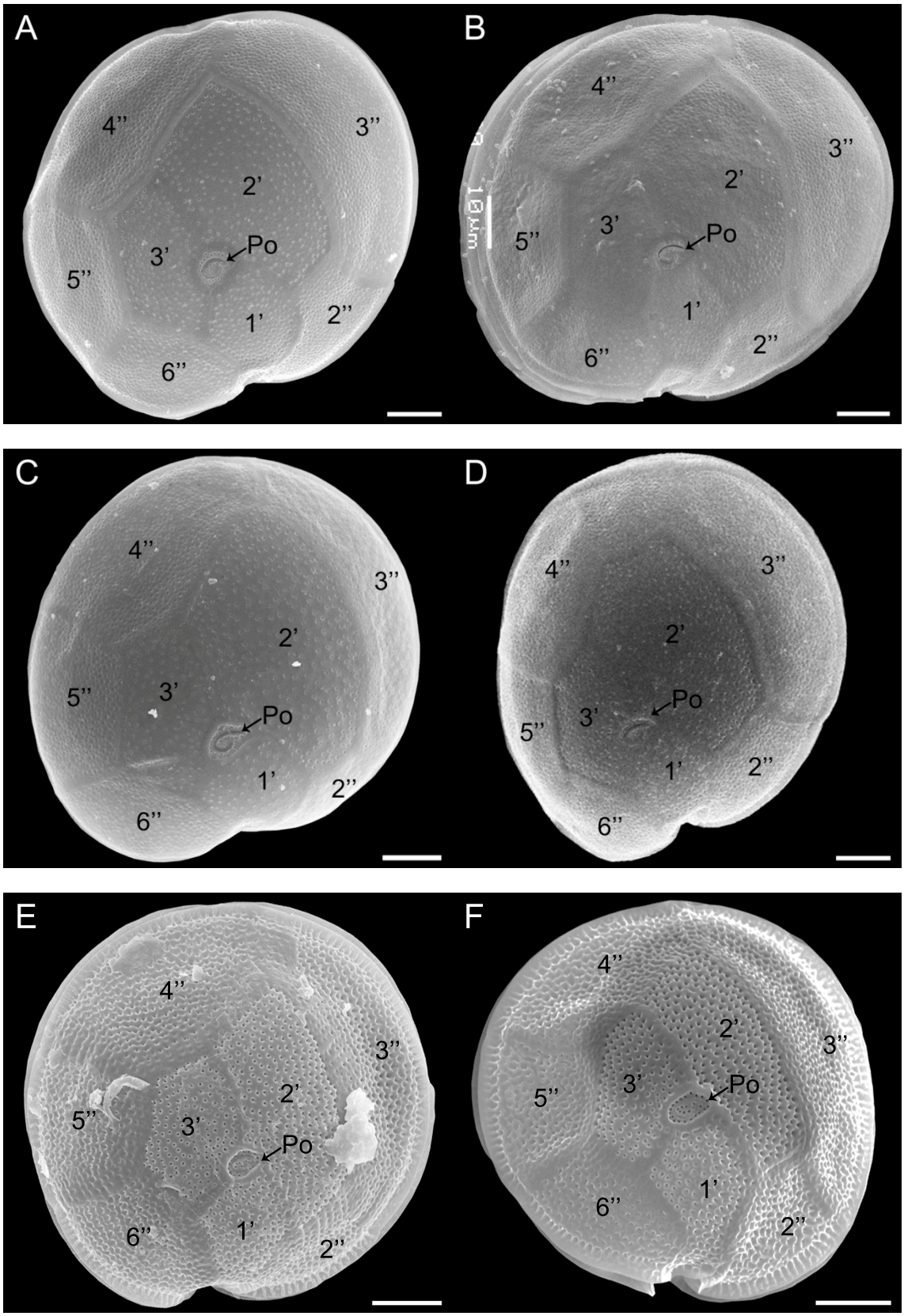

A
4''
3''
2'
Po
3'
5''
1'
2''
6''
B
4''
3''
2'
10 µm
5''
3'
Po
1'
6''
2''
C
4''
3''
2'
5''
3'
Po
1'
2''
6''
D
4''
3''
2'
Po
5''
3'
1'
2''
6''
E
4''
3''
2'
5''
3'
Po
6''
1'
2''
F
4''
2'
3''
5''
3'
Po
1'
6''
2''

Figure 6.

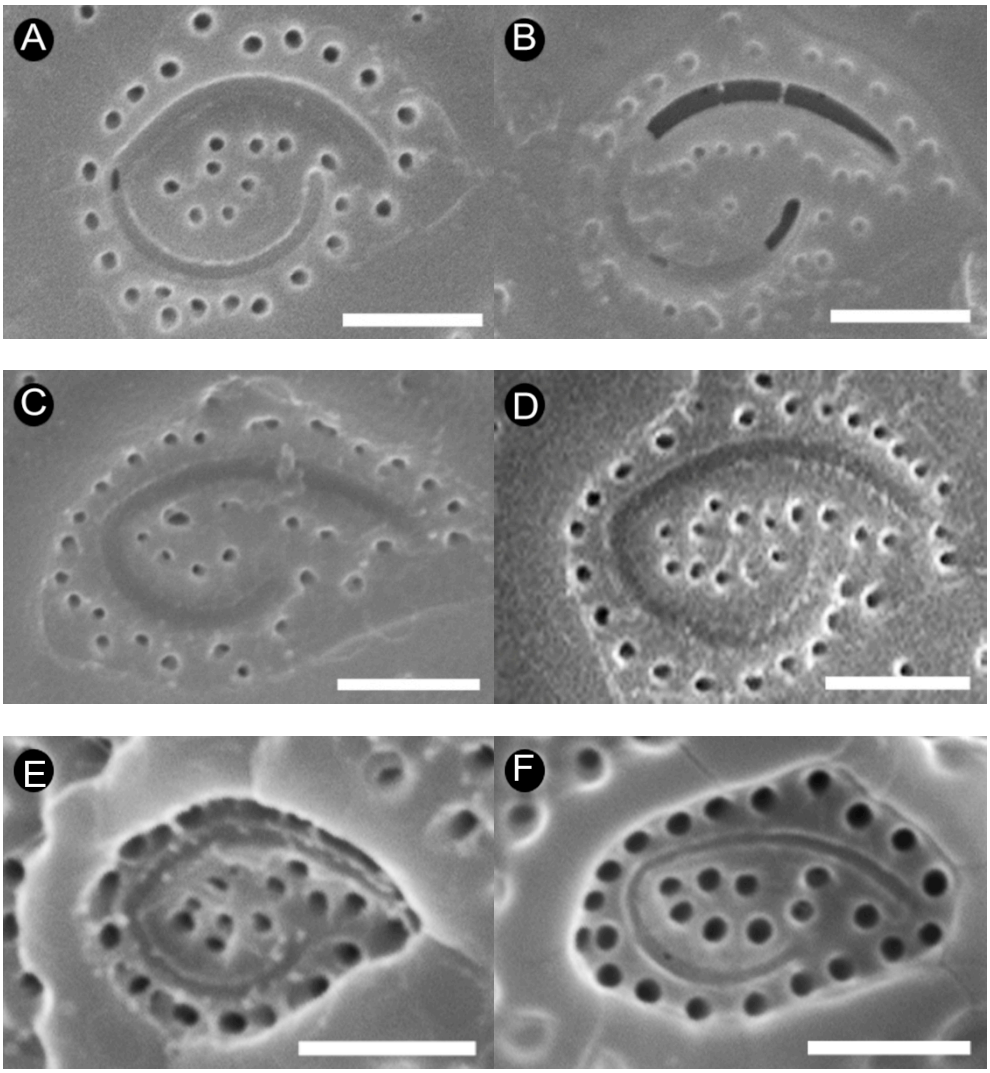
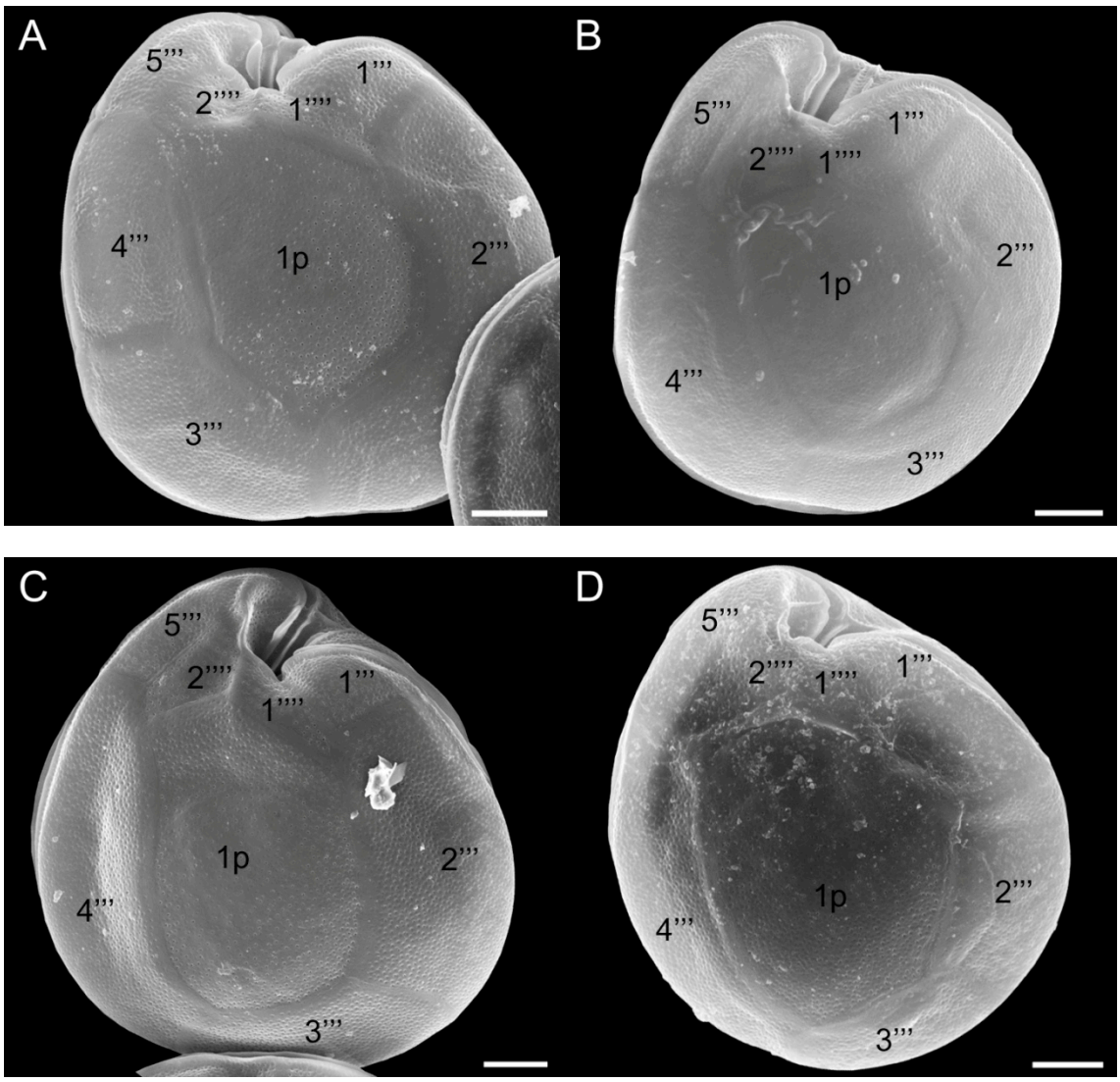
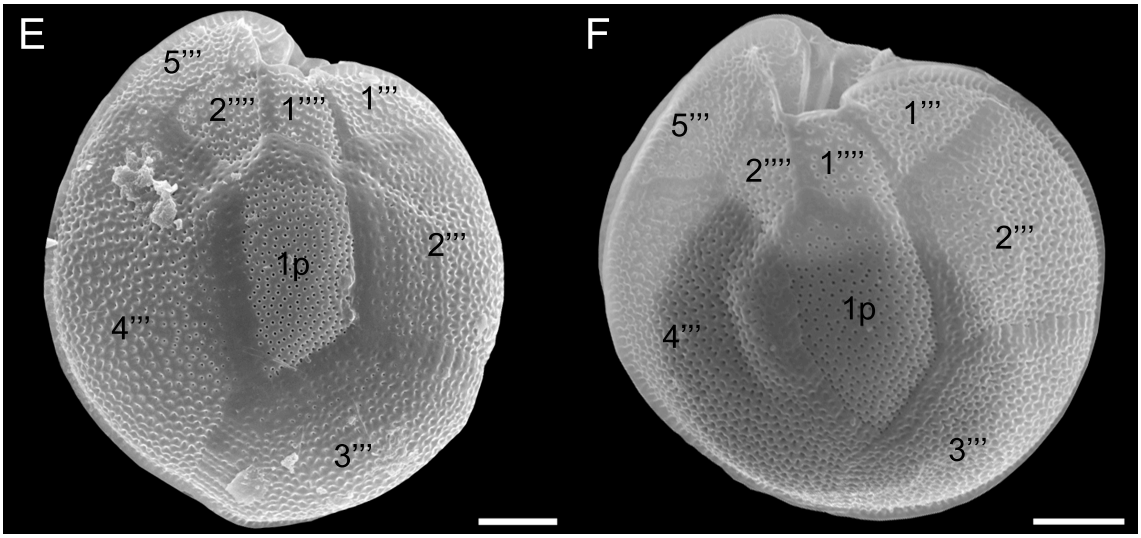


Figure 7.




E
5’’’
2’’’’
1’’’’
1’’’
2’’’
1p
4’’’
3’’’
F
5’’’
1’’’
2’’’’
1’’’’
2’’’
1p
4’’’
3’’’