

1 **LSU rDNA BASED RFLP ASSAYS FOR THE ROUTINE IDENTIFICATION OF**  
2 ***GAMBIERDISCUS* SPECIES**

3 Yihua Lyu<sup>a,b,§</sup>, Mindy L. Richlen<sup>a§,\*</sup>, Taylor R. Sehein<sup>a</sup>, Mireille Chinain<sup>c</sup>, Masao Adachi<sup>d</sup>,  
4 Tomohiro Nishimura<sup>d</sup>, Yixiao Xu<sup>a,e</sup>, Michael L. Parsons<sup>f</sup>, Tyler B. Smith<sup>g</sup>, Tianling Zheng<sup>h</sup>,  
5 Donald M. Anderson<sup>a</sup>

6 <sup>a</sup>Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

7 <sup>b</sup>South China Sea Environmental Monitoring Center, State Oceanic Administration, Guangzhou  
8 510300, China

9 <sup>c</sup>Laboratoire des Microalgues Toxiques, Institut Louis Malardé, UMR 241-EIO, BP 30, 98713  
10 Papeete Tahiti, French Polynesia

11 <sup>d</sup>Laboratory of Aquatic Environmental Science, Faculty of Agriculture, Kochi University, Otsu-  
12 200, Monobe, Nankoku, Kochi 783-8502, Japan

13 <sup>e</sup>Key Laboratory of Environment Change and Resources Use in Beibu Gulf, Ministry of  
14 Education, Guangxi Teachers Education University, Nanning 530001, China

15 <sup>f</sup>Coastal Watershed Institute, Florida Gulf Coast University, Fort Myers, Florida 33965, USA

16 <sup>g</sup>Center for Marine and Environmental Studies, University of the Virgin Islands, #2 John  
17 Brewers Bay, St Thomas, U.S. Virgin Islands 00802

18 <sup>h</sup>Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, School of  
19 Life Science, Xiamen University, Xiamen 361102, China

20 <sup>§</sup>These co-first authors contributed equally to this work

1 \*Corresponding author: Mindy L. Richlen, Woods Hole Oceanographic Institution, Woods Hole,  
2 MA 02543, USA; [mrichlen@whoi.edu](mailto:mrichlen@whoi.edu); Phone: 508-289-2552; Fax: 508-457-2027

3 **Keywords:** *Gambierdiscus*; ciguatera; RFLP; LSU rDNA

4  
5 **Abstract**

6 *Gambierdiscus* is a genus of benthic dinoflagellates commonly associated with ciguatera  
7 fish poisoning (CFP), which is generally found in tropical or sub-tropical regions around the  
8 world. Morphologically similar species within the genus can vary in toxicity; however, species  
9 identifications are difficult or sometimes impossible using light microscopy. DNA sequencing of  
10 ribosomal RNA genes (rDNA) is thus often used to identify and describe *Gambierdiscus* species  
11 and ribotypes, but the expense and time can be prohibitive for routine culture screening and/or  
12 large-scale monitoring programs. This study describes a restriction fragment length  
13 polymorphism (RFLP) typing method based on analysis of the large subunit ribosomal RNA  
14 gene (rDNA) that can successfully identify at least nine of the described *Gambierdiscus* species  
15 and two *Fukuyoa* species. The software programs DNAMAN 6.0 and Restriction Enzyme Picker  
16 were used to identify a set of restriction enzymes (*SpeI*, *HpyCH4IV*, and *TaqαI*) capable of  
17 distinguishing most of the known *Gambierdiscus* species for which DNA sequences were  
18 available. This assay was tested using *in silico* analysis and cultured isolates, and species  
19 identifications of isolates assigned by RFLP typing were confirmed by DNA sequencing. To  
20 verify the assay and assess intra-specific heterogeneity in RFLP patterns, identifications of 63  
21 *Gambierdiscus* isolates comprising ten *Gambierdiscus* species, one ribotype, and two *Fukuyoa*  
22 species were confirmed using RFLP typing, and this method was subsequently employed in the  
23 routine identification of isolates collected from the Caribbean Sea. The RFLP assay presented

1 here reduces the time and cost associated with morphological identification via scanning electron  
2 microscopy and/or DNA sequencing, and provides a phylogenetically sensitive method for  
3 routine *Gambierdiscus* species assignment.

4

## 5 **1. Introduction**

6 Ciguatera fish poisoning (CFP) is a human poisoning syndrome caused by the consumption of  
7 seafood contaminated with ciguatoxins. The genus *Gambierdiscus* represents a group of benthic  
8 dinoflagellates known to produce ciguatoxins (CTX); however, toxin production is variable  
9 among species (Holmes et al., 1991; Chinain et al., 2010). Incidences of CFP are more common  
10 in tropical and subtropical latitudes, which correspond to the endemic range of *Gambierdiscus*  
11 spp., and the prevalence of poisonings and abundances of *Gambierdiscus* spp. are often site-  
12 specific (Dickey and Plakas, 2010). *Gambierdiscus* dinoflagellates have been reported in tropical  
13 or sub-tropical regions around the world, including Okinawa in Japan (Nishimura et al., 2013),  
14 the South China Sea (Zhang et al., 2016), Hong Kong (Wong et al., 2005), Malaysia (Leaw et al.,  
15 2011), Thailand (Tawong et al., 2015), Texas, South Carolina, Hawaii, and Florida in U.S. (e.g.,  
16 Babinchak et al., 1986; CDC, 2006; Villareal et al., 2007; Rains and Parsons, 2015), French  
17 Polynesia (Chinain et al., 1991a, b), the Republic of Kiribati (Xu et al., 2014), Johnston Atoll  
18 (Richlen and Lobel, 2011), and other island nations in the Pacific (Lewis et al., 1991; Smith et al.,  
19 2016), Australia (Gillespie et al., 1985; Kohli et al., 2014; Kretzschmar et al., 2016), and the Red  
20 Sea (Saburova et al., 2013). More recently, *Gambierdiscus* was reported from temperate regions,  
21 including the Kermadec Islands, New Zealand (Rhodes et al., 2017), Japan (Kuno et al., 2010;  
22 Nishimura et al., 2013, 2014, 2016), Korea (Jeong et al., 2012), Canary Islands, Northeast

1 Atlantic (Fraga et al., 2011; 2014), Pakistan (Munir et al., 2011), the Gulf of Aqaba, Jordan  
2 (Saburova et al., 2013), the Northern Gulf of Mexico (Tester et al., 2013), and the Mediterranean  
3 Sea (Aligizaki and Nikolaidis, 2008). The increased prevalence of CFP in recent years may be  
4 attributed to multiple factors including improved awareness and/or reporting, expanding  
5 international trade in tropical fish species, climate change, increased anthropogenic activities,  
6 and the continued absence of affordable and accurate methods for detecting ciguatoxins in fish  
7 (Lehane and Lewis, 2000; Poon-King et al., 2004; Lewis, 2006; Chan et al., 2011).

8       Until 1995, all *Gambierdiscus* cells were recorded as *G. toxicus*; however, taxonomic  
9 studies carried out over the past two decades have identified 13 additional genetically and  
10 morphologically distinct species in the genus, including *G. australes*, *G. balechii*, *G. belizeanus*,  
11 *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. cheloniae*, *G. excentricus*, *G. lapillus*, *G.*  
12 *pacificus*, *G. polynesiensis*, *G. scabrosus*, *G. silvae*, as well as several ribotypes (Chinain et al.,  
13 1999a; Litaker et al., 2009; Kuno et al., 2010; Litaker et al., 2010; Fraga et al., 2011, 2014, 2016;  
14 Nishimura et al., 2013, 2014; Xu et al., 2014; Kretzschmar et al., 2016; Smith et al., 2016), and  
15 three closely related species recently reclassified as *Fukuyoa paulensis*, *F. ruetzleri* and *F.*  
16 *yasumotoi* (Gómez et al., 2015). *Gambierdiscus* toxin production is thought to be genetically  
17 determined, with significant variation in toxicity observed both within and among species (e.g.,  
18 Bomber et al., 1989; Chinain et al., 2010; Holmes et al., 1991; Sperr and Doucette, 1996;  
19 Pawlowicz et al., 2013). As *Gambierdiscus* populations found within a particular area can  
20 comprise multiple species that vary with respect to their toxicity, the species composition of  
21 blooms and particularly the presence of certain highly toxic species and/or strains have been  
22 suggested as playing a prominent role in CFP events and the severity of outbreaks (Holmes and

1 Lewis, 1994; Chinain et al., 1999b; Chinain et al., 2010). Further investigation of *Gambierdiscus*  
2 species biogeography and toxicity is still needed to support this hypothesis.

3 Ribosomal RNA gene sequences have been used to document species and strain diversity of  
4 *Gambierdiscus* populations globally and locally; however, DNA sequencing involves costly,  
5 labor-intensive procedures, and is impractical to apply on a large scale. More recently,  
6 community diversity profiling methods using quantitative PCR (qPCR) were developed for  
7 several *Gambierdiscus* species in field samples, including five Caribbean species (Vandersea et  
8 al., 2012) and four Japanese *Gambierdiscus* species/phylotypes (Nishimura et al., 2016). To  
9 contribute to the molecular tools currently available for characterizing *Gambierdiscus* species  
10 diversity, and specifically to aid in routine identification of cultures established from sampling  
11 sites in the Caribbean Sea, a restriction fragment length polymorphism (RFLP) assay based on  
12 the hypervariable D1-D2 region of the large subunit ribosomal RNA gene (LSU rDNA) was  
13 developed. To verify the assay and assess intra-specific heterogeneity in RFLP patterns, 63  
14 *Gambierdiscus* isolates comprising ten *Gambierdiscus* species, one ribotype, and two *Fukuyoa*  
15 species were identified using RFLP typing. The assay was subsequently and successfully  
16 employed in the routine identification of cultures established from samples collected from the  
17 Bahamas, St. Thomas, USVI, and the Florida Keys, FL, USA over the period of approximately  
18 two years. The assay presented here provides a rapid, phylogenetically sensitive, and inexpensive  
19 alternative to morphological identification via scanning electron microscopy (SEM) and/or DNA  
20 sequencing, and provides an alternative method for routine *Gambierdiscus* species assignment.  
21 The approach is also operationally simple, requiring basic molecular laboratory capabilities  
22 (PCR amplification and gel electrophoresis), and thus it could be useful in countries where DNA  
23 sequencing and/or SEM facilities are costly and/or unavailable.

1

## 2 **2. Materials and Methods**

### 3 *2.1 RFLP assay design and in silico testing*

4 For the assay design and *in silico* testing, sequences of the D1-D3 hypervariable region of the  
5 LSU rDNA from 12 *Gambierdiscus* species, one ribotype, and three *Fukuyoa* species were  
6 downloaded from the NCBI GenBank database, and the D1-D2 region was selected and used in  
7 subsequent analyses. Sequence data from this region was not available for *G. lapillus* and *G.*  
8 *balechii*, so these species were excluded from this analysis. The software programs DNAMAN  
9 6.0 (Lynnon Biosoft, Quebec, Canada) and Restriction Enzyme Picker (Collin and Rocap, 2007)  
10 were used to identify restriction enzymes that could distinguish these species. DNAMAN ENZ  
11 (Enzyme file), which contains 2523 enzymes, was used in the *in silico* analysis. The fragments  
12 with all cutters and ends were considered. Restriction Enzyme Picker was then used to optimize  
13 the enzyme combination, according to the principle of minimum fragments and enzyme number.  
14 *SpeI* was selected to distinguish *G. australes* from *G. carolinianus*, *TaqI* for *F. ruetzleri* and *F.*  
15 *yasumotoi*, and *HpyCH4IV* for the remaining *Gambierdiscus* species.

### 16 *2.2 Strain isolation and culture maintenance*

17 Live cultures established for the assay testing were isolated from St. Thomas, USVI, the Florida  
18 Keys, USA, and San Salvador, Bahamas (Parsons and Richlen, 2016). Additionally, cell pellets  
19 or DNA extracts were provided for several *Gambierdiscus* species from French Polynesia and  
20 Japan (*G. australes* and *G. scabrosus*). For culture establishment, individual *Gambierdiscus* or  
21 *Fukuyoa* spp. cells were isolated by micropipetting at 100× magnification, rinsed in sterile  
22 seawater, and established in 25% modified K medium (Morton and Norris, 1990). Clonal isolates

1 were subsequently transferred into tissue culture flasks and maintained in 100% modified K  
2 medium at 23°C, 32 psu, ~100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  of light, and 12h:12h light:dark photoperiod.  
3 A complete list of the isolates used in this study is provided in Table 1.

#### 4 2.3. RFLP analysis and sequencing

5 To verify the RFLP method, forty *Gambierdiscus* isolates, primarily from the Caribbean Sea,  
6 were identified using RFLP typing, and the species identity was confirmed using DNA  
7 sequencing. For these analyses, DNA was extracted from 1 ml of dense culture using the MoBIO  
8 PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the  
9 manufacturer's instructions and eluted in a final volume of 100  $\mu\text{l}$ . Partial fragments of the LSU  
10 rRNA gene were amplified from isolates using either the primers D1R and D2C (Scholin et al.,  
11 1994) or FD8 and RB (Chinain et al., 1999a). Each PCR reaction (25  $\mu\text{l}$ ) contained ~5 ng  
12 template DNA, 1 x PCR Buffer (500 mM KCl and 100 mM Tris-HCl, pH 8.3), 2 mM  $\text{MgCl}_2$ , 0.8  
13 mM dNTPs, 0.5  $\mu\text{M}$  of each primer, and 0.5 U of AmpliTaq DNA Polymerase (Applied  
14 Biosystems Inc., Foster City, CA, USA). Hot start PCR amplifications were performed in an  
15 Eppendorf Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) with the  
16 following cycling conditions: 94 °C for 4 min; then 35 cycles of 94 °C for 30 s, 57 °C for 1 min,  
17 72 °C for 2 min, and a final extension of 72 °C for 10 min. PCR products were visualized by  
18 electrophoresis on 2% TAE agarose gel to verify that the PCR reaction was successful, and to  
19 assess the uncut PCR product size. PCR products used in the RFLP assay were then purified  
20 using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany).

21 RFLP reactions (25  $\mu\text{l}$ ) contained 4  $\mu\text{l}$  of purified D1-D2 PCR product (variable, but  
22 generally ranging from ~100-200 ng), 18  $\mu\text{l}$  water, 2.5  $\mu\text{l}$  1x CutSmart® Buffer, and 0.5  $\mu\text{l}$  (5 U)

1 of *SpeI* and *HpyCH4IV* (New England Biolabs, Inc., Ipswich, MA, USA). Samples were covered  
2 with plastic wrap to prevent evaporation and incubated at 37 °C for 15 min in the Eppendorf  
3 thermal cycler. The temperature was reduced to 4 °C and 0.5 µl (50 U) *TaqαI* (New England  
4 Biolabs, Inc.) was added to each reaction, then samples were incubated at 65 °C for 15 min  
5 followed by 80 °C for 20 min to deactivate the enzymes. RFLP digestion products, along with a  
6 100 bp DNA ladder, were separated by electrophoresis on 2% TAE agarose gel at 75V for 1.5  
7 hours.

8 For DNA sequencing, unpurified PCR products were cloned into the pGEM T Easy Vector  
9 (Promega, Madison, WI, USA). Clones were screened with plasmid primers M13F and M13R,  
10 and sequenced in both the forward and reverse direction (Eurofins MWG Operon, Ebersberg,  
11 Germany). DNA sequences were aligned in Geneious Pro 8.1 (Biomatters, Auckland, NZ), and  
12 the consensus sequences were compared with those in GenBank using BLAST sequence  
13 similarity searches (NCBI). Restriction maps used to analyze DNA sequences from isolates  
14 exhibiting aberrant RFLP patterns were created with Geneious Pro 8.1.

### 15 3. Results

16 *In silico* analyses identified a combination of three enzymes (*SpeI*, *HpyCH4IV*, and *TaqαI*) that  
17 produced fragments unique to the *Gambierdiscus* species examined, as well as two  
18 morphologically similar *Fukuyoa* species. *SpeI* distinguished *G. australes* and *G. carolinianus*,  
19 *TaqαI* distinguished *F. ruetzleri* and *F. yasumotoi*, and *HpyPYCH4IV* differentiated among the  
20 remaining *Gambierdiscus* species. *Gambierdiscus toxicus* did not contain any restriction  
21 recognition sites, and generated a PCR product of ca. 726 bp. Enzyme recognition sites are listed  
22 in Table 2, and expected fragment sizes from the *in silico* analysis are provided in Table 3.



1 Ten *Gambierdiscus* species, one ribotype (*Gambierdiscus* ribotype 2), and two *Fukuyoa*  
2 species were analyzed using this assay to verify the fragment sizes generated by the *in silico*  
3 analysis (Fig. 1). These analyses showed that the fragment bands produced by the RFLP  
4 digestion were comparable to those predicted by *in silico* analysis. All fragments greater than  
5 100 base pairs were clearly visible in the agarose gel; however, fragment bands smaller than 100  
6 base pairs were sometimes faint or difficult to visualize (Fig. 1). Efforts to improve the  
7 visualization of these smaller bands by increasing the amount of digested product used in gel  
8 electrophoresis (up to 12  $\mu$ l) were unsuccessful, as the smaller fragments still appeared faint  
9 regardless of the amount analyzed (data not shown).

10 The RFLP assay was then tested with multiple *Gambierdiscus* isolates from the Caribbean  
11 Sea and the Pacific to further assess the assay's consistency and accuracy, including potential  
12 intra-specific variability. DNA sequence data were also collected from these isolates to verify  
13 species identifications assigned by RFLP typing. Consensus sequences were compared with  
14 those deposited in GenBank using BLAST sequence similarity searches (National Centre for  
15 Biotechnology Information, NCBI) to confirm the species identification. Results from both the  
16 RFLP digestion and DNA sequencing are listed in Table 1, and a subset shown in Fig. 2.  
17 Fragment sizes were generally uniform within species (exceptions described below), and the  
18 species identifications from the RFLP assay correctly corresponded to previously assigned  
19 species identifications. Undigested PCR product was occasionally observed in digest patterns of  
20 *G. carpenteri* (Fig. 1 and Fig. 2G, H) and *G. australes* (Fig. 1 and Fig. 2I), but this did not  
21 interfere with species identification.

22 *Gambierdiscus belizeanus*, *G. silvae*, and *Gambierdiscus* ribotype 2 exhibited consistent, but  
23 similar, fragment sizes that required further evaluation (Fig. 3A). Analyzing the band size of the

1 uncut D1-D2 PCR product enabled identification of *G. belizeanus*, as this species consistently  
2 produced two bands that were easily distinguished using gel electrophoresis (Fig. 3B).  
3 Distinguishing between *G. silvae* and *Gambierdiscus* ribotype 2, however, was more difficult.  
4 *Gambierdiscus silvae* has a shorter D1-D2 sequence length (ca. 688 bp) compared to that of  
5 *Gambierdiscus* ribotype 2 (ca. 744 bp), but this size difference was not readily apparent on an  
6 agarose gel unless these taxa were analyzed side by side (Fig. 3B).

7       In order to investigate the intra-specific uniformity in RFLP patterns for species distributed  
8 in both the Pacific Ocean and Caribbean Sea, isolates of *G. caribaeus* and *G. carpenteri* from  
9 French Polynesia were analyzed and their RFLP profiles compared with conspecific isolates  
10 from St. Thomas, USVI. Restriction site analysis of LSU rDNA sequences (D1-D2 region)  
11 collected from these isolates was also carried out to determine if differences in RFLP profiles  
12 could be attributed to sequence heterogeneity. Pacific isolates of both species exhibited variation  
13 in RFLP patterns, which was consistently observed in multiple digests of these particular strains.  
14 For example, the ~278 bp digestion fragment that was clearly visible in restriction profiles of *G.*  
15 *caribaeus* isolates from the Caribbean (Fig. 2A) appeared to be absent from RFLP profiles of the  
16 Pacific *G. caribaeus* isolates from French Polynesia (Fig. 2B). Restriction site analysis of D1-D2  
17 sequences of the *G. caribaeus* isolate NH-1 from French Polynesia showed that certain clones  
18 exhibited a single base change directly adjacent to the *Hpy*CH4IV recognition site, which may  
19 have resulted in the loss of this restriction site in these strains (Supplementary Fig. S1).  
20 Information about this phenomenon is scarce, but the loss of recognition sites due to point  
21 mutations in flanking bases has been reported previously (Klein et al., 1991). Despite this  
22 difference, the Pacific isolates were readily identified as *G. caribaeus* based on the other  
23 observed fragments. Additionally, RFLP analysis of the two Pacific isolates of *G. carpenteri*

1 generated banding patterns that differed from conspecific isolates from the Caribbean (Fig. 2G),  
2 and also from each other (Fig. 2H). Both isolates produced the expected ca. 233 bp fragment;  
3 however, additional bands observed did not match the species-specific RFLP pattern (Fig. 2G,  
4 H). Sequence analysis confirmed that both isolates are indeed *G. carpenteri* (Supplementary Fig.  
5 S2), but species identification could not be assigned with the RFLP assay due to these unique  
6 banding patterns. Restriction site analysis of the D1-D2 sequences of these isolates showed that  
7 isolate NH-2 contained internal deletions (Supplementary Fig. S2). These particular sequences  
8 were ~60 bp shorter than the full-length sequences. Additionally, some of the sequences  
9 obtained from these isolates included base changes that produced additional recognition sites for  
10 the enzymes used in this assay.

## 11 **Discussion**

12 Over the past two decades, significant progress has been made in identifying and describing the  
13 considerable species and strain diversity within the *Gambierdiscus* genus, thus advancing our  
14 knowledge of the biogeography and community composition of *Gambierdiscus* populations. The  
15 toxin producing capabilities of these newly described species and ribotypes are not fully known,  
16 although prior laboratory studies have shown that toxin production is highly variable among  
17 species (e.g., Chinain et al., 1999a) and strains (e.g., Holmes et al., 1994). As multiple  
18 *Gambierdiscus* species can co-exist within a particular reef ecosystem, information on the  
19 community diversity and the prevalence of toxin-producing species and strains is an important  
20 part of assessing and understanding spatial and temporal trends in the prevalence of toxic fish  
21 and cases of ciguatera. The goal of this study was to develop a rapid and low-cost method for  
22 routine species identification that can be used in conjunction with monitoring programs, either as  
23 a screening method prior to the selection of species and strains for further study, or in

1 combination with other methods of community diversity profiling (e.g., qPCR; Vandersea et al.,  
2 2012; Nishimura et al., 2016). This assay was employed in the routine identification of isolates  
3 from the Caribbean Sea, but is also capable of distinguishing several *Gambierdiscus* species  
4 commonly observed in the Pacific.

5 Because RFLP analysis is operationally straightforward, comparatively inexpensive, and  
6 does not require specialized equipment, it has obvious advantages compared to other methods  
7 such as DNA sequencing, which for *Gambierdiscus* requires bacterial cloning of PCR products  
8 to distinguish pseudogenes (Richlen and Barber, 2005), and may not be practical for the  
9 identification of large numbers of cultured strains. RFLP assays provide a rapid and reliable  
10 means for screening large numbers of cultures, and have been widely used for species  
11 identifications and in the study of the community structure of many different groups of  
12 microorganisms such as fungi and algae, including taxa responsible for HABs (Scholin et al.,  
13 1994; Chang et al., 2006; Dickie and FitzJohn, 2007). For example, RFLP assays for  
14 *Alexandrium* spp. were described by Scholin and Anderson (1994), Scholin et al. (1996), and  
15 Adachi et al. (1994), based on multiple restriction enzyme cleavage of small subunit (SSU)  
16 rRNA gene, LSU rRNA gene, and 5.8S rDNA-ITS regions, respectively. RFLP profiling has also  
17 been used to distinguish *Alexandrium affine* and *A. margalefii* from Bahía Concepción, Mexico  
18 (Band-Schmidt et al., 2003). These methods have been used with great success to identify  
19 several toxin-producing species responsible for paralytic shellfish poisoning (PSP), which  
20 prompted the approach outlined here.

21 In this study, the restriction enzymes *SpeI*, *HpyCH4IV*, and *TaqαI* were selected for the  
22 assay based on successful *in silico* analysis, and this combination was tested using DNA extracts  
23 of ten *Gambierdiscus* species, one ribotype, and two morphologically similar *Fukuyoa* species.

1 Subsequent to these analyses, we used RFLP profiling to analyze a culture collection comprising  
2 63 isolates to assess intra-specific length heterogeneity and RFLP pattern uniformity. In these *in*  
3 *silico* and laboratory analyses, the selected enzyme combination generated apparent and unique  
4 DNA fragment patterns for all species tested, with the exception of *G. silvae* and *Gambierdiscus*  
5 ribotype 2, which exhibited similar RFLP patterns (Fig. 3A), and required longer electrophoresis  
6 duration (>1.5 h) to separate them (Fig. 3B). Examination of the uncut PCR product aided in  
7 distinguishing these groups, as the D1-D2 region exhibits length heterogeneity that can be  
8 visualized on an agarose gel (ca. 688 bp and 744 bp for *G. silvae* and *Gambierdiscus* ribotype 2,  
9 respectively; see Fig. 3B). However, this size difference was not readily apparent on an agarose  
10 gel unless these taxa were analyzed side by side. Increasing the duration of gel electrophoresis or  
11 separation on an acrylamide gel may aid in resolving these species, or an alternative means of  
12 identification may occasionally be required. Several quality control (QC) procedures to ensure  
13 that the assay is functioning properly can also be employed, and include: (1) analyzing uncut  
14 PCR product using gel electrophoresis to ensure that PCR amplification was successful, and to  
15 assess uncut PCR product length; (2) analyzing both uncut and digested DNA on the same gel to  
16 better distinguish length heterogeneity of the uncut DNA and identify undigested PCR product;  
17 (3) measure and standardize the PCR product concentration used in each digestion reaction (for  
18 labs with access to a nanodrop or some other means of analyzing DNA concentration); (4)  
19 including one or more positive controls (i.e., DNA extraction from an identified culture) in PCR  
20 amplifications and RFLP digestions; and (5) including one or more positive controls (digested  
21 PCR product) along with unknowns in the gel electrophoresis analysis. To the extent feasible,  
22 multiple isolates of each species were tested, but for some species only one DNA extract was

1 analyzed due to the limited availability of these isolates, and recently described species were not  
2 tested due to the unavailability of cultures.

3 During these analyses, the smaller fragments (<100 bp) used to help distinguish *G.*  
4 *carolinianus*, *G. polynesiensis*, and *G. toxicus* were often faint (Fig. 1), possibly due to the low  
5 resolution of agarose electrophoresis, differences in DNA concentrations, and limitation of visual  
6 observation. Increasing the digestion volume (up to 12  $\mu$ l) did not improve the appearance of  
7 these fragments; however, using polyacrylamide rather than agarose gels would likely improve  
8 the resolution. Fortunately, differences in banding patterns were such that this limitation did not  
9 affect the assay's ability to effectively discriminate among these species.

10 Additionally, RFLP digests of *G. carpenteri* and *G. caribaeus* strains from the Pacific  
11 (French Polynesia) produced fragment patterns that differed from the Caribbean isolates (Figs.  
12 2A-B, 2G-H), despite multiple repeats of the digest. One of the fragments present in restriction  
13 profiles of *G. caribaeus* isolates from the Caribbean appeared to be absent from profiles of the  
14 Pacific isolates (Figs. 2A-B). *Gambierdiscus carpenteri* from the Pacific also exhibited banding  
15 patterns that were different from the Caribbean isolates, and also from each other. Three bands  
16 (ca. 443, 320, and 233 bp) were observed in digests of isolate Rik-5 and three distinct bands (ca.  
17 385, 233, and 115 or 118 bp) were observed in digests of NH-2 (Fig. 2H). Based on restriction  
18 site analysis of the D1-D2 sequences of these isolates, these RFLP patterns are likely due to the  
19 presence of nucleotide substitutions that either eliminated restriction sites (*G. caribaeus*,  
20 Supplementary Fig. S1), or produced additional recognition sites for the enzymes used in this  
21 assay (*G. carpenteri*, Supplementary Fig. S2). Pseudogenes containing internal deletions are  
22 well-documented in *Gambierdiscus* spp. (Richlen and Barber, 2005) and may have also  
23 contributed to these aberrant RFLP patterns. With the exception of these isolates, aberrant

1 patterns were infrequently observed; however, the variation we observed in these globally  
2 distributed and closely related species illustrates that an alternative confirmation method may  
3 occasionally be needed to confirm an isolate's identity.

4 The RFLP approach offers several advantages over other approaches to species  
5 identification (DNA sequencing, SEM) and the analysis of community diversity, although the  
6 method does require some laboratory skill and resources, and there are biases inherent in using  
7 this approach to assess community diversity. As cells must be isolated from field samples and  
8 established in culture, the survival of particular strains over others may skew the perception of  
9 species composition. The RFLP also requires both a PCR and digestion step, but the cost to  
10 perform these reactions is far lower than both qPCR and DNA sequencing costs. Another  
11 advantage of this method is its sensitivity. For example, this method readily discriminates  
12 groups separated by very low phylogenetic distance (e.g, *G. toxicus* and *G. pacificus*; *G.*  
13 *caribaeus* and *G. carpenteri*). Overall, the RFLP method greatly benefits labs with culturing  
14 facilities that are interested in a low cost, phylogenetically sensitive, and rapid screening  
15 approach to identify *Gambierdiscus* isolates. The approach is also operationally simple, requiring  
16 basic molecular laboratory capabilities (PCR amplification and gel electrophoresis), making the  
17 method useful in countries where DNA sequencing facilities and/or SEM are costly and/or  
18 unavailable.

19 Following its development, this method was successfully used in conjunction with DNA  
20 sequencing for the identification of cultures established during monthly sampling in St. Thomas,  
21 USVI, and the Florida Keys, USA, as well as isolates established from San Salvador, Bahamas.  
22 These cell isolation and culture establishment activities were carried out routinely as part of a  
23 broader program to assess the *Gambierdiscus* population dynamics, community composition, and

1 growth physiologies of *Gambierdiscus* species/ribotypes at these study locations. The  
2 development of this assay was motivated by the labor and expense associated with traditional  
3 methods of species identification of the large numbers of cultures established during this  
4 research (e.g., DNA sequencing and morphological analysis). The enzyme combination used in  
5 this assay proved to be a sensitive and effective method for distinguishing most of the described  
6 *Gambierdiscus* species, and complements other existing, validated methods for species  
7 identification and the analysis of community diversity.

## 8 **Conclusions**

9 Here the development of a RFLP assay that effectively distinguishes at least nine *Gambierdiscus*  
10 species, and two morphologically similar *Fukuyoa* species is described. This method was tested  
11 using cultures established during monthly monitoring to assess *Gambierdiscus* abundance and  
12 toxicity in St. Thomas, USVI, the Bahamas, and the Florida Keys, USA, and with isolates from  
13 French Polynesia and Japan. This LSU rDNA-based RFLP assay readily distinguished most of  
14 the known *Gambierdiscus* species, and was successfully used over a period of two years to  
15 identify isolates established from field sampling in the Caribbean. Where possible, multiple  
16 isolates of each species were examined, many of which exhibited intra-specific uniformity in  
17 their electrophoretic patterns; however, additional work is still needed to investigate the  
18 interference of pseudogenes, and to better document intra-specific sequence heterogeneity  
19 observed in Pacific versus Caribbean strains of *G. caribaeus* and *G. carpenteri*. Nonetheless, this  
20 assay proved to be an effective method for routine identification of *Gambierdiscus* species, and  
21 could supplement or in some instances replace current methods for the analysis of laboratory  
22 cultured isolates.



1 **Acknowledgments**

2 Funding for this study was provided by the U.S. National Oceanic and Atmospheric  
3 Administration ECOHAB program (CiguaHAB; Cooperative Agreement NA11NOS4780060,  
4 NA11NOS4780028), the China Scholarship Council and Natural Science Foundation of China  
5 (No. 41606137, 41606136), and the Guangxi Natural Science Foundation  
6 (2015GXNSFCA139003, 2016GXNSFBA380037). We are very grateful to Dr. Deana Erdner  
7 for providing several isolates from St. Thomas to use in our methods testing. We also thank  
8 Chris Loeffler, Amy Henry, Lauren Henry, Amanda Ellsworth, Ashley Brandt, and Alex Leynse  
9 for sample collection and for helping to establish and maintain the isolates used in these analyses,  
10 and two anonymous editors for their review and constructive critique. This is ECOHAB  
11 publication number 880.

1 **Tables**

2 **Table 1.** Isolate name and geographic origin of *Gambierdiscus* and *Fukuyoa* spp. used for RFLP assay testing, and comparison  
 3 between RFLP typing results and identification based on DNA sequencing or alternative method. Percent identity levels based on  
 4 BLAST sequence similarity searches in GenBank are shown in parentheses. In the interest of simplifying the assay description and  
 5 results, the first four letters of each species name (shown in alphabetical order) is used to represent each *Gambierdiscus* and *Fukuyoa*  
 6 species, except for *Gambierdiscus* ribotype 2 (Ribo2).

<b>Isolates</b>	<b>Geographic Origin</b>	<b>Abbreviation</b>	<b>Species identification based on DNA sequencing or alternative method</b>	<b>RFLP Recognition</b>
<b>BB Apr 11-11</b>	St. Thomas, USVI	Cari1	<i>G. caribaeus</i> (100%)	<i>G. caribaeus</i>
<b>BB May 10-12</b>	St. Thomas, USVI	Cari2	<i>G. caribaeus</i> (99%)	<i>G. caribaeus</i>
<b>BP Aug 08</b>	St. Thomas, USVI	Cari3	<i>G. caribaeus</i> (99%)	<i>G. caribaeus</i>
<b>HGB7</b>	Florida Keys, FL, USA	Cari4	<i>G. caribaeus</i> (100%)	<i>G. caribaeus</i>
<b>LKH4</b>	Florida Keys, FL, USA	Cari5	<i>G. caribaeus</i> (99%)	<i>G. caribaeus</i>
<b>Tenn10</b>	Florida Keys, FL, USA	Cari6	<i>G. caribaeus</i> (100%)	<i>G. caribaeus</i>
<b>STT_Cari6</b>	St. Thomas, USVI	Cari7	<i>G. caribaeus</i> (99%)	<i>G. caribaeus</i>
<b>STT_Cari19</b>	St. Thomas, USVI	Cari8	<i>G. caribaeus</i> (100%)	<i>G. caribaeus</i>
<b>NH-1</b>	Nuku-Hiva, Marquesas, French Polynesia	Cari9	<i>G. caribaeus</i> (100%)	<i>G. caribaeus</i>
<b>Rik-1</b>	Mangareva, Gambier, French Polynesia	Cari10	<i>G. caribaeus</i> <sup>a</sup>	<i>G. caribaeus</i>

---

<b>BB May 10-11</b>	St. Thomas, USVI	Caro1	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>FC Apr 11-2</b>	St. Thomas, USVI	Caro2	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>BP May 10-5</b>	St. Thomas, USVI	Caro3	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>LKH10</b>	Florida Keys, FL, USA	Caro4	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>GHCG2-C6</b>	San Salvador, Bahamas	Caro5	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>TRL26</b>	Florida Keys, FL, USA	Caro6	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>GHCG2-A6</b>	San Salvador, Bahamas	Caro7	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>GHCG2-B8</b>	San Salvador, Bahamas	Caro8	<i>G. carolinianus</i> (99%)	<i>G. carolinianus</i>
<b>CCMP399</b>	St. Barthelemy Island	Beli1	<i>G. belizeanus</i> <sup>b</sup>	<i>G. belizeanus</i>
<b>FC Dec 10-13</b>	St. Thomas, USVI	Beli2	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>BP Apr 11-7</b>	St. Thomas, USVI	Beli3	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>BP Mar 10-18</b>	St. Thomas, USVI	Beli4	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>BP Mar 10-22</b>	St. Thomas, USVI	Beli5	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>BP Mar 10-25</b>	St. Thomas, USVI	Beli6	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>BP Mar 10-31</b>	St. Thomas, USVI	Beli7	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>BP Mar 10-7</b>	St. Thomas, USVI	Beli8	<i>G. belizeanus</i> (99%)	<i>G. belizeanus</i>
<b>MUR-4</b>	Moruroa, Gambier, French Polynesia	Paci1	<i>G. pacificus</i> <sup>c</sup>	<i>G. pacificus</i>
<b>Hao1 (or HO-91)</b>	Hao, Tuamotu, French Polynesia	Paci2	<i>G. pacificus</i> <sup>c</sup>	<i>G. pacificus</i>
<b>Tub ET1</b>	Tubuai, Australes, French Polynesia	Paci3	<i>G. pacificus</i> <sup>a</sup>	<i>G. pacificus</i>
<b>BP Apr 11-6</b>	St. Thomas, USVI	Ribo21	<i>G. ribotype 2</i> (99%)	<i>G. ribotype 2</i>

---

<b>SH Dec 10-10</b>	St. Thomas, USVI	Ribo22	<i>G. ribotype 2 (99%)</i>	<i>G. ribotype 2</i>
<b>SH Dec 10-12</b>	St. Thomas, USVI	Ribo23	<i>G. ribotype 2 (99%)</i>	<i>G. ribotype 2</i>
<b>TRL29</b>	Florida Keys, FL, USA	Ribo24	<i>G. ribotype 2 (100%)</i>	<i>G. ribotype 2</i>
<b>HGB</b>	Florida Keys, FL, USA	Yasu	<i>F. yasumotoi (94%)</i>	<i>F. yasumotoi</i>
<b>HGB6</b>	Florida Keys, FL, USA	Carp1	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>KML1</b>	Florida Keys, FL, USA	Carp2	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>TPH12</b>	Florida Keys, FL, USA	Carp3	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>STT_Carp5</b>	St. Thomas, USVI	Carp4	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>STT_Carp8</b>	St. Thomas, USVI	Carp5	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>STT_Carp9</b>	St. Thomas, USVI	Carp6	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>STT_Carp11</b>	St. Thomas, USVI	Carp7	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>STT_Carp24</b>	St. Thomas, USVI	Carp8	<i>G. carpenteri (99%)</i>	<i>G. carpenteri</i>
<b>Rik-5</b>	Mangareva, Gambier, French Polynesia	Carp9	<i>G. carpenteri (98%)</i>	Inconclusive
<b>NH-2</b>	Nuku-Hiva, Marquesas, French Polynesia	Carp10	<i>G. carpenteri (99%)</i>	Inconclusive
<b>PO</b>	Tahiti, Society, French Polynesia	Aust1	<i>G. australes</i> <sup>a</sup>	<i>G. australes</i>
<b>RAV-1</b>	Raivavae, Australes, French Polynesia	Aust2	<i>G. australes</i> <sup>a</sup>	<i>G. australes</i>
<b>G3-93</b>	Mangareva, Gambier, French Polynesia	Aust3	<i>G. australes</i> <sup>a</sup>	<i>G. australes</i>
<b>S080911_1</b>	Kutsu, Kochi, Japan	Aust4	<i>G. australes</i> <sup>e</sup>	<i>G. australes</i>
<b>ISC5G</b>	Touzato, Ishigaki Island, Okinawa, Japan	Aust5	<i>G. australes</i> <sup>e</sup>	<i>G. australes</i>
<b>I080606_1</b>	Sawada Beach, Irabu Island, Okinawa, Japan	Aust6	<i>G. australes</i> <sup>e</sup>	<i>G. australes</i>

<b>Rai1</b>	Raivavae, Australes, French Polynesia	Poly1	<i>G. polynesiensis</i> <sup>c</sup>	<i>G. polynesiensis</i>
<b>Rik-8</b>	Mangareva, Gambier, French Polynesia	Poly2	<i>G. polynesiensis</i> <sup>a</sup>	<i>G. polynesiensis</i>
<b>RG-92</b>	Rangiroa, Tuamotu, French Polynesia	Poly3	<i>G. polynesiensis</i> <sup>c</sup>	<i>G. polynesiensis</i>
<b>TB-92</b>	Tubuai, French Polynesia	Poly4	<i>G. polynesiensis</i> <sup>e</sup>	<i>G. polynesiensis</i>
<b>GTT-1</b>	Tahiti, Society, French Polynesia	Toxi1	<i>G. toxicus</i> <sup>a</sup>	<i>G. toxicus</i>
<b>Rik-13</b>	Mangareva, Gambier, French Polynesia	Toxi2	<i>G. toxicus</i> <sup>a</sup>	<i>G. toxicus</i>
<b>HIT-0</b>	Tahiti, French Polynesia	Toxi3	<i>G. toxicus</i> <sup>c</sup>	<i>G. toxicus</i>
<b>CCMP 3143</b>	Carrie Bow Cay, Belize	Ruez	<i>F. ruetzleri</i> <sup>b</sup>	<i>F. ruetzleri</i>
<b>BP Mar 10-23</b>	St. Thomas, USVI	Silv1	<i>G. silvae</i> (100%)	<i>G. silvae</i>
<b>FC May 10-9</b>	St. Thomas, USVI	Silv2	<i>G. silvae</i> (99%)	<i>G. silvae</i>
<b>SH Apr 11-1</b>	St. Thomas, USVI	Silv3	<i>G. silvae</i> (99%)	<i>G. silvae</i>
<b>TRL23</b>	Florida Keys, FL, USA	Silv4	<i>G. silvae</i> (99%)	<i>G. silvae</i>
<b>M080828_3</b>	Muroto Promontory, Kochi, Japan	Scab	<i>G. scabrosus</i> <sup>d</sup>	<i>G. scabrosus</i>

1

2 <sup>a</sup> Isolate from culture collection maintained by the Institut Louis Malardé, Tahiti, French Polynesia

3 <sup>b</sup> Isolate from culture collection maintained by the National Center for Marine Algae and Microbiota at Bigelow Laboratory, East Boothbay, ME,  
4 USA

5 <sup>c</sup> see Chinain et al. (2010)

6 <sup>d</sup> see Nishimura et al. (2013)

7 <sup>e</sup> see Chinain et al. (1999a)

8

9

10

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16

**Table 2.** Recognition sites of the restriction enzymes *SpeI*, *HpyCH4IV*, and *TaqαI*.

Enzyme	<i>SpeI</i>	<i>HpyCH4IV</i>	<i>TaqαI</i>
Recognition site	A/CTAGT	A/CGT	T/CGA

1 **Table 3.** Fragment sizes for 12 *Gambierdiscus* species, one ribotype, and three *Fukuyoa* species.  
 2 Abbreviated species names are represented by the first four letters of each species name except  
 3 for *Gambierdiscus* ribotype 2 (Ribo2). Fragments are listed from 5' to 3' end of each D1-D2  
 4 LSU rDNA sequence.

<i>Gambierdiscus</i> spp.	Abbreviation	Uncut PCR product size	Restriction fragment sizes (bp)
<i>G. australes</i>	Aust	687	493, 194
<i>G. belizeanus</i>	Beli	770, ~640*	617, 153
<i>G. caribaeus</i>	Cari	676	389, 278, 184, 98
<i>G. carolinianus</i>	Caro	691	635, 56
<i>G. carpenteri</i>	Carp	676	443, 233
<i>G. cheloniae</i>	Chel	712	659, 53
<i>G. excentricus</i>	Exce	741	500, 262, 49
<i>G. pacificus</i>	Paci	763	487, 153, 123
<i>G. polynesiensis</i>	Poly	706	626, 80
<i>G. scabrosus</i>	Scab	778	512, 276
<i>G. silvae</i>	Silv	688	573, 115
<i>G. toxicus</i>	Toxi	726	726
<i>Gambierdiscus</i> ribotype 2	Ribo2	744	609, 135
<i>F. ruetzleri</i>	Ruet	747	530, 142, 75
<i>F. paulensis</i>	Paul	708	432, 103, 98, 40, 35
<i>F. yasumotoi</i>	Yasu	697	434, 188, 75

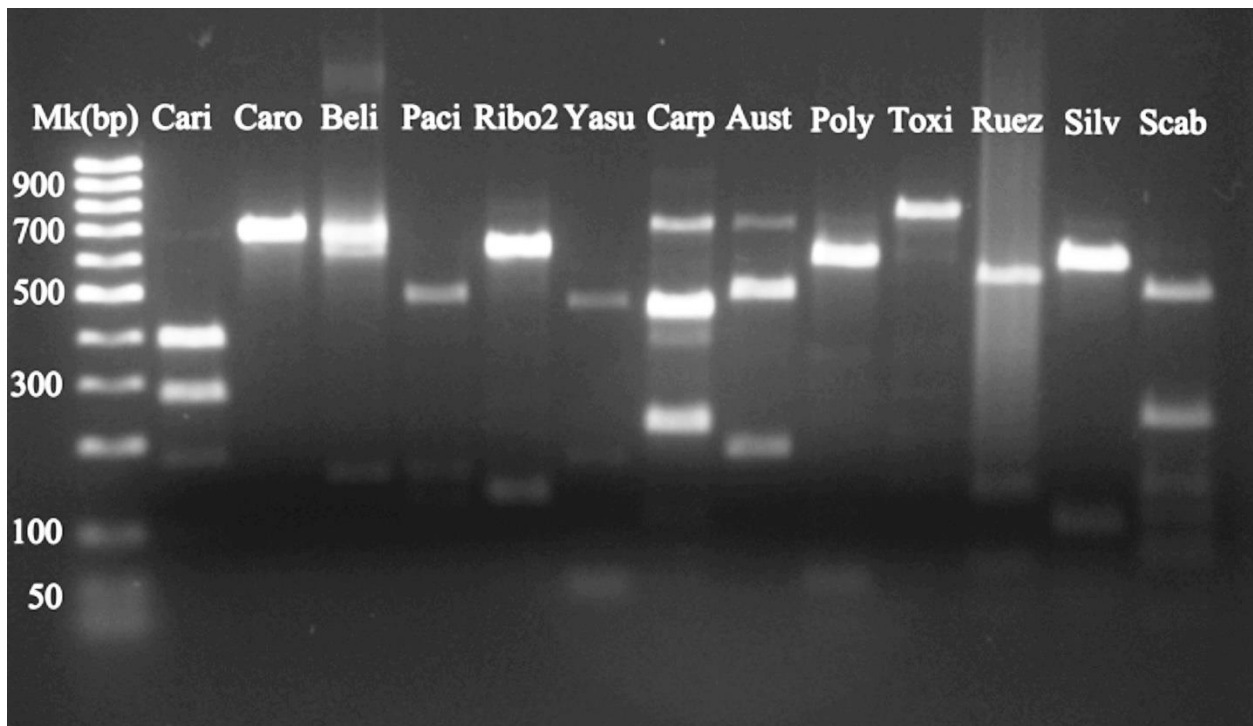
5  
 6 \* Smaller fragment size estimated from agarose gel.

7

1 **Figures**

2 **Figure 1.** RFLP profiles of LSU rDNA (D1-D2 hypervariable regions) from ten *Gambierdiscus*  
3 species, one ribotype, and two *Fukuyoa* species. Cari: *G. caribaeus* (BP Aug 08), Caro: *G.*  
4 *carolinianus* (GHCG2-C6), Beli: *G. belizeanus* (CCMP399), Paci: *G. pacificus* (Tub ET1),  
5 Ribo2: *Gambierdiscus* ribotype 2 (SH Dec 10-10), Yasu: *F. yasumotoi* (HGB), Carp: *G.*  
6 *carpenteri* (HGB6), Aust: *G. australes* (RAV-1), Poly: *G. polynesiensis* (Rai1), Toxi: *G. toxicus*  
7 (GTT-1), Ruez: *F. ruetzleri* (CCMP 3143), Silv: *G. silvae* (SH Apr 11-1), Scab: *G. scabrosus*  
8 (M080828\_3), Mk: DNA marker.

9



10

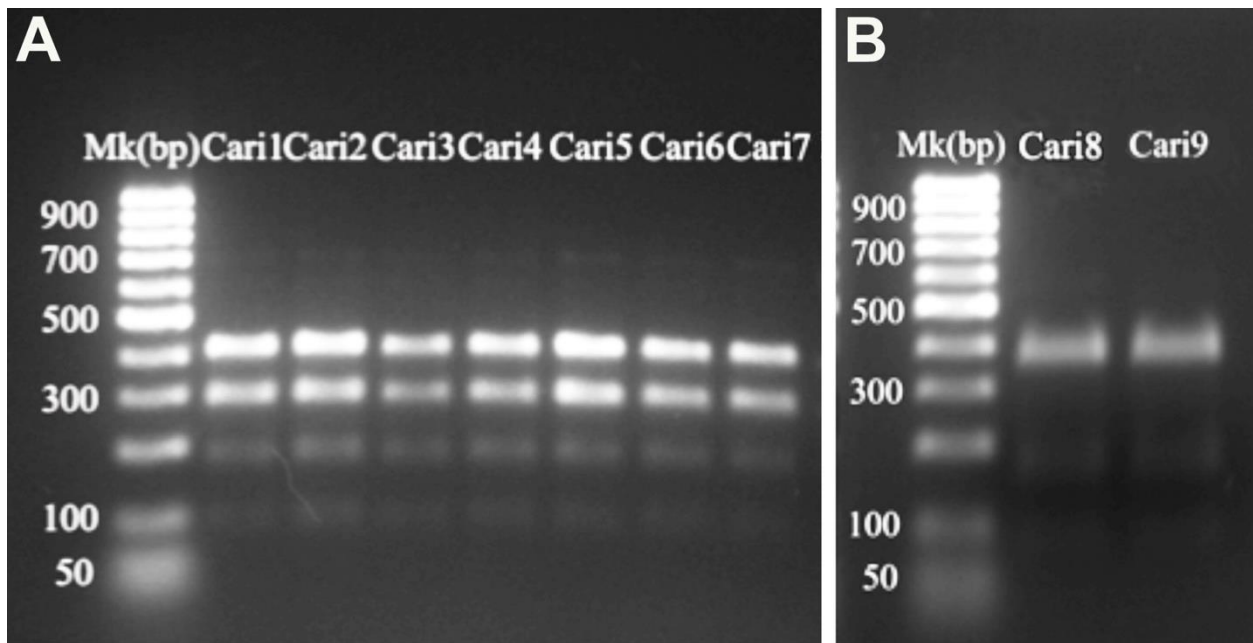
11

12



1

2 **Figure 2.** RFLP profiles of LSU rDNA (D1-D2 hypervariable regions) of conspecific strains. A:  
3 *G. caribaeus* from Caribbean Sea (Cari1~7), B: *G. caribaeus* from French Polynesia (Cari8~9),  
4 C: *G. carolinianus* (Caro1~8), D: *G. belizeanus* (Beli1~8), E: *G. pacificus* (Paci1~3), F:  
5 *Gambierdiscus* ribotype 2 (Ribo21~4), G: *G. carpenteri* from the Caribbean Sea (Carp1~8), H:  
6 Pseudogene-containing *G. carpenteri* from French Polynesia (Carp9 and 10), I: *G. australes* (Aust1~6),  
7 J: *G. polynesiensis* (Poly1~4), K: *G. toxicus* (Toxi1~3), L: *G. silvae* (Silv1~4). See Table 1 for  
8 additional information regarding the isolates used in each assay, including the isolate  
9 abbreviations above each lane.

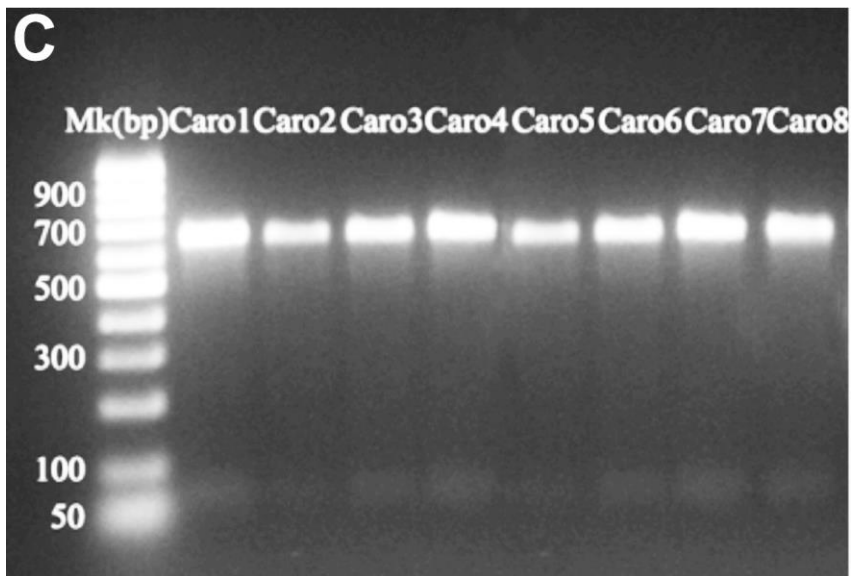


10

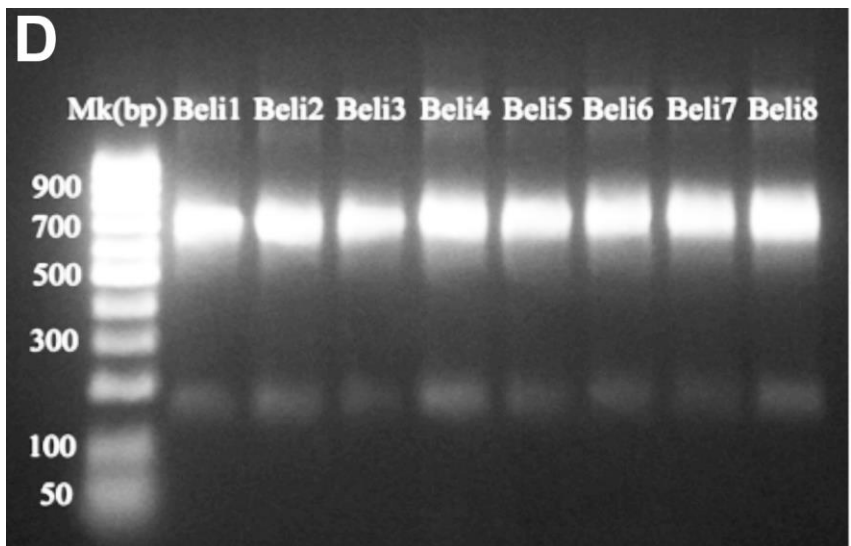
11

12

13



1



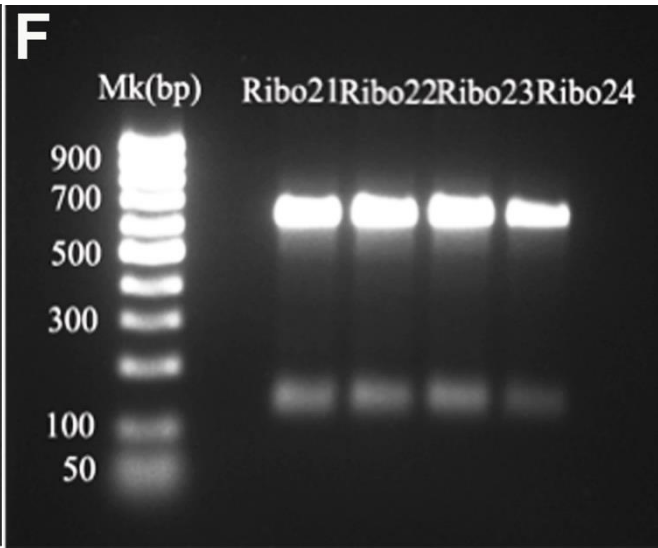
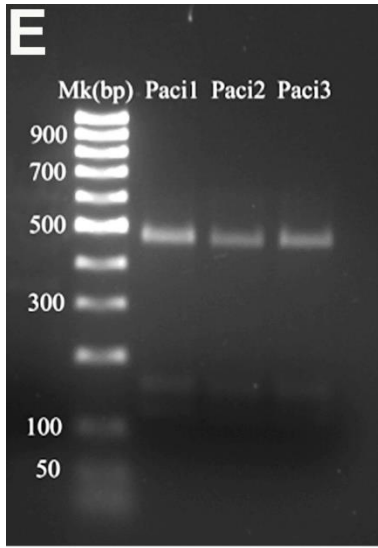
2

3

4

5

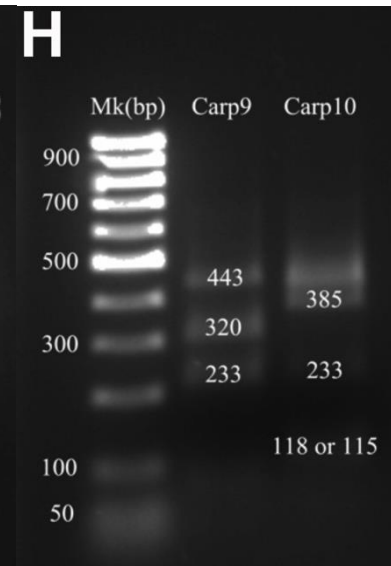
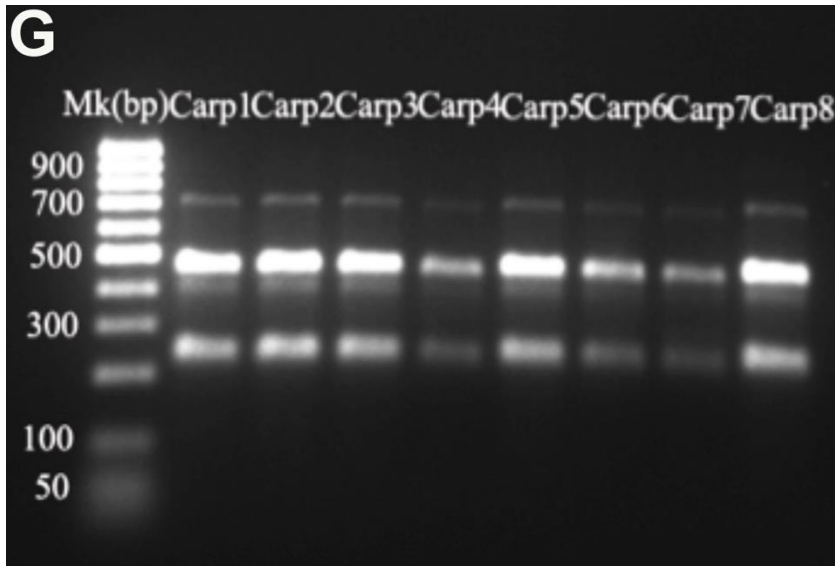
6



1

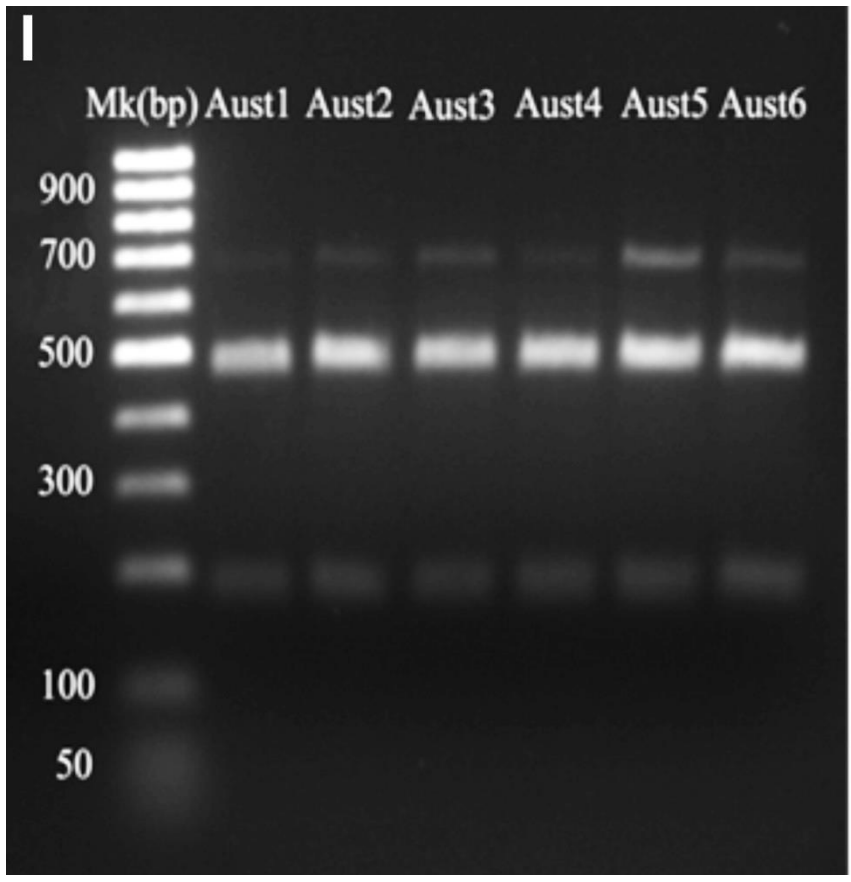
2

3



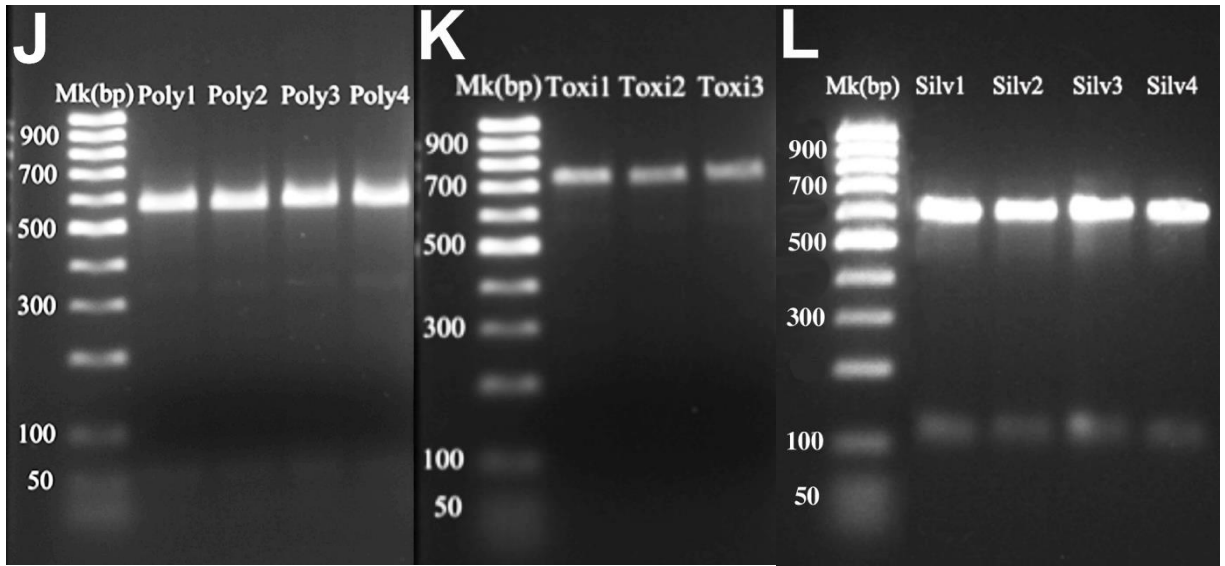
4

5



1

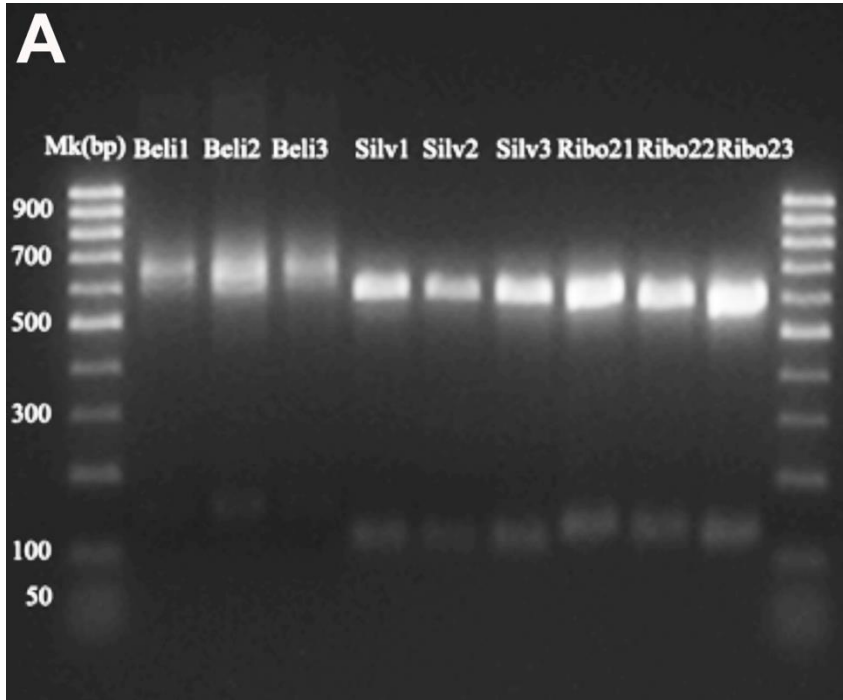
2



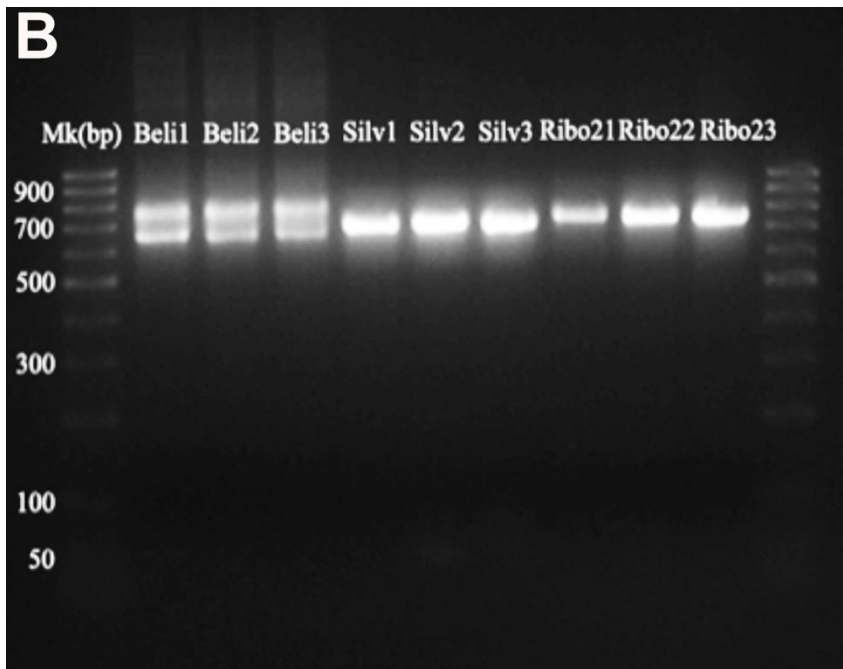
3

4

1 **Figure 3.** RFLP profiles (A) and uncut PCR product (B) of LSU rDNA (D1-D2 hypervariable  
2 regions) from *Gambierdiscus belizeanus*, *G. silvae*, and *Gambierdiscus* ribotype 2. See Table 1  
3 for additional information regarding the isolates used in each assay.

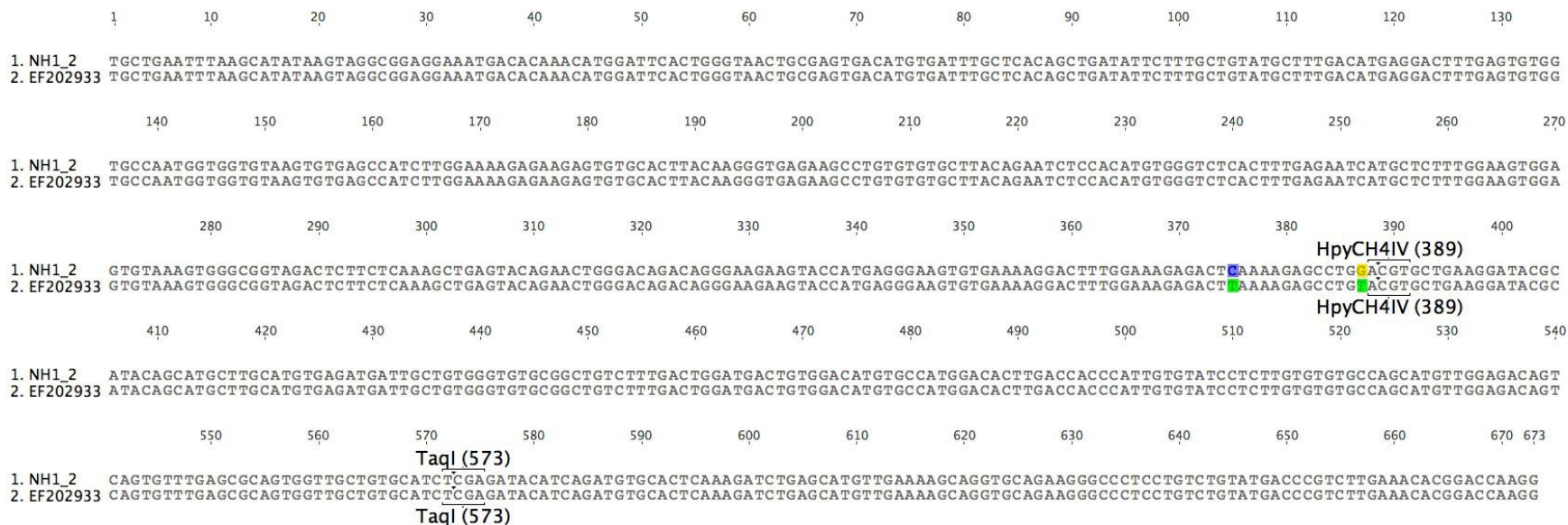


4



5

- 1 **Supplementary Figure S1.** Alignment of D1-D2 LSU rRNA gene sequence from *G. caribaeus* isolate NH-1 from French Polynesia,
- 2 along with a *G. caribaeus* sequence from GenBank (accession no. EF202933), annotated with known restriction sites.



- 3
- 4
- 5
- 6
- 7



1 **References**

- 2 Adachi, M., Sako, Y., Ishida, Y., 1994. Restriction fragment length polymorphism of ribosomal  
3 DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species  
4 (Dinophyceae). *Journal of Phycology*, 30, 857-863.
- 5 Aligizaki, K., Nikolaidis, G., 2008. Morphological identification of two tropical dinoflagellates  
6 of the genera *Gambierdiscus* and *Sinophysis* in the Mediterranean Sea. *Journal of Biological*  
7 *Research-Thessaloniki*, 9, 75-82.
- 8 Babinchak, J.A., Jollow, D.J., Voegtline, M.S., Higerd, T.B., 1986. Toxin production by  
9 *Gambierdiscus toxicus* isolated from the Florida Keys. *Marine Fisheries Review*, 48, 53-56.
- 10 Band-Schmidt, C.J., Lilly, E.L., Anderson, M.D., 2003. Identification of *Alexandrium affine* and  
11 *A. margalefii* (Dinophyceae) using DNA sequencing and LSU rDNA-based RFLP-PCR assays.  
12 *Phycologia*, 42, 261-268.
- 13 Bomber, J.W., Tindall, D.R., Miller, D.M., 1989. Genetic variability in toxin potencies among  
14 seventeen clones of *Gambierdiscus toxicus* (Dinophyceae). *Journal of Phycology*, 25: 617-625.
- 15 Centers for Disease Control and Prevention (CDC), 2006. Ciguatera Fish Poisoning-Texas, 1998,  
16 and South Carolina, 2004. *MMWR Morb. Mortal. Wkly. Rep.* 55, pp. 935-937.
- 17 Chang, W., Um, Y., Holoman, T.R.P., 2006. Polycyclic aromatic hydrocarbon (PAH)  
18 degradation coupled to methanogenesis. *Biotechnology Letter*, 28, 425-430.
- 19 Chan, W.H., Mak, Y.L., Wu, J.J., Jin, L, Sit, W.H., Lam, J.C., Sadovy de Mitcheson, Y., Chan,  
20 L.L., Lam, P.K., Murphy, M.B., 2011. Spatial distribution of ciguateric fish in the Republic of  
21 Kiribati. *Chemosphere*, 1, 117-123.



1 Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S.,  
2 2010. Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus*  
3 *polynesiensis* (Dinophyceae) in culture. *Toxicon*, 56, 739-750.

4 Chinain, M., Faust, M.A., Pauillac, S., 1999a. Morphology and molecular analyses of three toxic  
5 species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp nov., *G. australes*, sp nov., and *G.*  
6 *polynesiensis*, sp nov. *Journal of Phycology*, 6, 1282-1296.

7 Chinain, M., Germain, M., Deparis, X., Pauillac, S., Legrand, A.M., 1999b. Seasonal abundance  
8 and toxicity of the dinoflagellate *Gambierdiscus* spp. (Dinophyceae), the causative agent of  
9 ciguatera in Tahiti, French Polynesia. *Marine Biology*, 135, 259-267.

10 Collins, R.E., Rocap, G., 2007. REPK: an analytical web server to select restriction  
11 endonucleases for terminal restriction fragment length polymorphism analysis. *Nucleic Acids*  
12 *Research*, 35 (Database issue): W58-W62; doi:10.1093/nar/gkm384

13 Dickey, R.W., Plakas, S.M., 2010. Ciguatera: A public health perspective. *Toxicon*, 2, 123-136.

14 Dickie, I.A., FitzJohn, R.G., 2007. Using terminal restriction fragment length polymorphism (T-  
15 RFLP) to identify mycorrhizal fungi: a methods review. *Mycorrhiza*, 17, 259-270.

16 Fraga, S., Rodríguez, F., Caillaud, A., Diogene, J., Raho, N., Zapata, M., 2011. *Gambierdiscus*  
17 *excentricus* sp nov (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE  
18 Atlantic Ocean). *Harmful Algae*, 11, 10-22.

19 Fraga, S., Rodríguez, F., 2014. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean)  
20 with Description of *Gambierdiscus silvae* sp. nov., a New Potentially Toxic Epiphytic Benthic  
21 Dinoflagellate. *Protist*, 165, 839-853.

1 Fraga, S., Rodríguez, F., Riobó, P., Bravo, I., 2016. *Gambierdiscus balechii* sp. nov.  
2 (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean).  
3 *Harmful Algae*, 58, 93-105.

4 Gillespie, N.C., Holmes, M.J., Burke, J.B., Doley, J., 1985. Distribution and periodicity of  
5 *Gambierdiscus toxicus* in Queensland, Australia, in: Anderson D.M., White A.W., Baden D.G.  
6 (Ed.), *Toxic Dinoflagellates*. Elsevier, New York, pp. 183-188.

7 Gómez, F., Qiu, D., Lopes, R.M., Lin, S., 2015. *Fukuyoa paulensis* gen. et sp. nov., a new genus  
8 for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). *PLoS ONE*, 10,  
9 e0119676.

10 Holmes, M.J., Lewis, R.J., Poli, M.A., Gillespie, N.C., 1991. Strain dependent production of  
11 ciguatoxin precursors (gambiertoxins) by *Gambierdiscus toxicus* (Dinophyceae) in culture.  
12 *Toxicon*, 6, 761-765.

13 Holmes, M.J., Lewis, R.J., Sellin, M., Street, R., 1994. The origin of ciguatera in Platypus Bay,  
14 Australia. *Mem. Queensland Mus. Brisbane*, 34, 505-512.

15 Holmes, M.J., Lewis, R.J., 1994 The origin of ciguatera. *Memoirs of the Queensland Museum*,  
16 34, 497-504.

17 Jeong, H.J., Lim, A.S., Jang, S.H., Yih, W.H., Kang, N.S., Lee, S.Y., Yoo, Y.D., Kim, H.S.,  
18 2012 First report of the epiphytic dinoflagellate *Gambierdiscus caribaeus* in the temperate  
19 waters off Jeju island, Korea: morphology and molecular characterization. *Journal of Eukaryotic*  
20 *Microbiology*, 59, 637-650.

1 Klein, D., Dodson, A.E., Tabor, D.E., Cederbaum, S.D., Grody, W.W., 1991. Effect of an  
2 adjacent base on detection of a point mutation by restriction enzyme digestion. *Somatic Cell and*  
3 *Molecular Genetics*, 17, 369-375.

4 Kohli, G.S., Murray, S.A., Neilan, B.A., Rhodes, L.L., Harwood, D.T., Smith, K.F., Meyer, L.,  
5 Capper, A., Brett, S. and Hallegraeff, G.M., 2014. High abundance of the potentially maitotoxic  
6 dinoflagellate *Gambierdiscus carpenteri* in temperate waters of New South Wales, Australia.  
7 *Harmful Algae*, 39, 34-145.

8 Kretzschmar, A.L., Verma, A., Harwood, D.T., Hoppenrath, M., Murray, S., 2016.  
9 Characterization of *Gambierdiscus lapillus* sp. nov. (Gonyaulacales, Dinophyceae): a new toxic  
10 dinoflagellate from the Great Barrier Reef (Australia). *Journal of Phycology*. DOI:  
11 10.1111/jpy.12496

12 Kuno, S., Kamikawa, R., Yoshimatsu, S., Sagara, T., Nishio, S., Sako, Y., 2010 Genetic diversity  
13 of *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) in Japanese coastal areas.  
14 *Phycological Research*, 1, 44-52.

15 Leaw, C.P., Lim, P.T., Tan, T.H., Tuan-Halim, T.N., Cheng, K.W., Ng, B.K., Usup, G., 2011  
16 First report of the benthic dinoflagellate, *Gambierdiscus belizeanus* (Gonyaulacales:  
17 Dinophyceae) for the east coast of Sabah, Malaysian Borneo. *Phycological Research*, 59, 143-  
18 146.

19 Lehane, L., Lewis, R., 2000 Ciguatera: recent advances but the risk remains. *International*  
20 *Journal of Food Microbiology*, 61, 91-125.

21 Lewis, R.J., 1991 Socioeconomic impacts and management ciguatera in the Pacific. *Bulletin de*  
22 *la Societe de pathologie exotique* (1990), 85, no. 5 (Pt 2), 427-434.

1 Lewis, R.J., 2006 Ciguatera: Australian perspectives on a global problem. *Toxicon*, 7, 799-809.

2 Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Chinain, M., Holmes, M.J., Holland,  
3 W.C., Tester, P.A., 2009. Taxonomy of *Gambierdiscus* including four new species,  
4 *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri* and  
5 *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). *Phycologia*, 48, 344-390.

6 Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Nau, A.W., Holland, W.C., Chinain,  
7 M., Holmes, M.J., Tester, P.A., 2010. Global distribution of ciguatera causing dinoflagellates in  
8 the genus *Gambierdiscus*. *Toxicon*, 5, 711-730.

9 Munir, S., Siddiqui, P.J.A., Morton, S.L., 2011. The occurrence of the ciguatera fish poisoning  
10 producing dinoflagellate genus *Gambierdiscus* in Pakistan waters. *Algae*, 26, 317-325.

11 Morton, S.L., Norris, D.R., 1990. The role of temperature, salinity, and light on the seasonality  
12 of *Prorocentrum lima*, In: Graneli, E. (Ed.), Toxic marine phytoplankton. Elsevier, New York,  
13 pp. 201-205.

14 Nishimura, T., Hariganeya, N., Tawong, W., Sakanari, H., Yamaguchi, H., Adachi, M. 2016.  
15 Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate  
16 *Gambierdiscus* spp. (Gonyaulacales) in coastal areas of Japan. *Harmful Algae*, 52, 11-22.

17 Nishimura, T., Sato, S., Tawong, W., Sakanari, H., Uehara, K., Shah, M.M.R., Suda, S.,  
18 Yasumoto, T., Taira, Y., Yamaguchi, H., Adachi, M., 2013. Genetic diversity and distribution of  
19 the ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in coastal areas of Japan.  
20 PLoS ONE, 8, e60882.

1 Nishimura, T., Sato, S., Tawong, W., Sakanari, H., Yamaguchi, H., Adachi, M., 2014.  
2 Morphology of *Gambierdiscus scabrosus* sp. Nov. (Gonyaulacales): a new epiphytic toxic  
3 dinoflagellate from coastal areas of Japan. *Journal of Phycology*, 50, 506-514.

4 Parsons, M.L., Richlen, M.L., 2016. An overview of ciguatera fish poisoning in the Bahamas. In:  
5 Erdman, R. and R. Morrison (Editors). *The 15<sup>th</sup> Symposium on the Natural History of the*  
6 *Bahamas*. Gerace Research Centre, San Salvador, Bahamas, p. 1-10.

7 Pawlowicz, R., Darius, H.T., Cruchet, P., Rossi, F.F., Caillaud, A., Laurent, D., Chinain, M.,  
8 2013. Evaluation of seafood toxicity in the Australes archipelago (French Polynesia), using the  
9 neuroblastoma cell based assay. *Food Additives & Contaminants: Part A*, 3, 567-586.

10 Poon-king, C.M., Chen, A., Poon-King T., 2004. Ciguatera fish poisoning in industrial ship  
11 crewmembers: a retrospective study in a seaport general practice in Trinidad and Tobago. *West*  
12 *Indian Medical Journal*, 53, 220-226.

13 Rains, L.K., Parsons, M.L., 2015. *Gambierdiscus* species exhibit different epiphytic behaviors  
14 toward a variety of macroalgal hosts. *Harmful Algae*, 49, 29-39.

15 Rhodes, L., Papiol, G.G., Smith, K., Harwood, T., 2014. *Gambierdiscus* cf. *yasumotoi*  
16 (Dinophyceae) isolated from New Zealand's sub-tropical northern coastal waters. *New Zealand*  
17 *Journal of Marine and Freshwater Research*, 2, 203-310.

18 Rhodes, L.L., Smith, K.F., Verma, A., Murray, S., Harwood, D.T., Trnski, T., 2017. The  
19 dinoflagellate genera *Gambierdiscus* and *Ostreopsis* from subtropical Raoul Island and North  
20 Meyer Island, Kermadec Islands. *New Zealand Journal of Marine and Freshwater Research*, 1-15.

1 Richlen, M.L., Barber, P.H., 2005. A technique for the rapid extraction of microalgal DNA from  
2 single live and preserved cells. *Molecular Ecology Notes*, 5, 688-691.

3 Richlen, M.L., Lobel, P.S., 2011. Effects of depth, habitat, and water motion on the abundance  
4 and distribution of ciguatera dinoflagellates at Johnston Atoll, Pacific Ocean. *Marine Ecology*  
5 *Progress Series*, 421, 51-66.

6 Richlen, M.L., Morton, S.L., Barber, P.H., Lobel, P.S., 2008. Phylogeography, morphological  
7 variation and taxonomy of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae).  
8 *Harmful algae*, 7, 614-629.

9 Saburova, M., Polikarpov, I., Al-Yamani F., 2013. New records of the genus *Gambierdiscus* in  
10 marginal seas of the Indian Ocean. *Marine Biodiversity Records*, 6, e91.

11 Scholin, C.A., Anderson, D.M., 1994. Identification of group and strain-specific genetic markers  
12 for globally distributed *Alexandrium* (Dinophyceae). II. RFLP analysis of LSU rRNA genes.  
13 *Journal of Phycology*, 30, 999-1011.

14 Scholin, C.A., Anderson, D.M., 1996. LSU rDNA-Based RFLP assays for discriminating species  
15 and strains of *Alexandrium* (Dinophyceae). *Journal of Phycology*, 32, 1022-1035.

16 Smith, K.F., Rhodes, L., Verma, A., Curley, B.G., Harwood, D.T., Kohli, G.S., Solomona, D.,  
17 Rongo, T., Munday, R., Murray, S.A., 2016. A new *Gambierdiscus* species (Dinophyceae) from  
18 Rarotonga, Cook Islands: *Gambierdiscus cheloniae* sp. nov. *Harmful Algae*, 60, 45-56.

19 Sperr, A.E., Doucette, G.J., 1996. Variation in growth rate and ciguatera toxin production among  
20 geographically distinct isolates of *Gambierdiscus toxicus*, in: Yasumoto, T., Oshima, Y., Fukuyo,  
21 Y. (Eds.), *Harmful and toxic algal blooms*. United Nations Educational, Scientific and Cultural  
22 Organization, Paris, France, pp. 309-312.

1 Tawong, W., Nishimura, T., Sakanari, H., Sato, S., Yamaguchi, H., Adachi, M. 2015.  
2 Characterization of *Gambierdiscus* and *Coolia* (Dinophyceae) isolates from Thailand based on  
3 morphology and phylogeny. *Phycological Research*, 63, 125-133.

4 Tester, P., Vandersea, M.W., Buckel, C.A., Kibler, S.R., Holland, W.C., Davenport, E.D., Clark,  
5 R.D., Edwards, K.F., Taylor, J.C., Vander Pluym, J.L., Hickerson, E.L., Litaker, R.W., 2013  
6 *Gambierdiscus* (Dinophyceae) species diversity in the Flower Garden Banks National Marine  
7 Sanctuary, Northern Gulf of Mexico, USA. *Harmful Algae*, 29, 1-9.

8 Vandersea, M.W., Kibler, S.R., Holland, W.C., Tester, P.A., Schultz, T.F., Faust, M.A., Holmes,  
9 M.J., Chinain, M. Litaker RW, 2012 Development of semi-quantitative PCR assays for the  
10 detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae). *Journal of*  
11 *Phycology*, 48, 902-915.

12 Villareal, T.A., Hanson, S., Qualia, S., Jester, E.L.E., Granade H.R., Dickey R.W., 2007.  
13 Petroleum production platforms as sites for the expansion of ciguatera in the northwestern Gulf  
14 of Mexico. *Harmful Algae*, 6, 253-259.

15 Wong, C.K.K., Hung, P., Lee, K.L., Kam, K.M.M., 2005. Study of an outbreak of ciguatera fish  
16 poisoning in Hong Kong. *Toxicon*, 46, 563-571.

17 Xu, Y., Richlen, M.L., Morton, S.L., Mak, M., Chan, L.L., Tekiau, A., Anderson, D.M., 2014.  
18 Distribution, abundance and diversity of *Gambierdiscus* spp. from a ciguatera-endemic area in  
19 Marakei, Republic of Kiribati. *Harmful Algae*, 34, 56-68.

20 Zhang, H, Wu, Z., Cen, J., Li, Y., Wang, H., Lu, S., 2016. First report of three benthic  
21 dinoflagellates, *Gambierdiscus pacificus*, *G. australes* and *G. caribaeus* (Dinophyceae), from  
22 Hainan Island, South China Sea. *Phycological Research*, 64: 259-73.