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

Yihua Lyu[a,b,§], Mindy L. Richlen[a§,*], Taylor R. Sehein[a], Mireille Chinain[c], Masao Adachi[d], Tomohiro Nishimura[d], Yixiao Xu[a,e], Michael L. Parsons[f], Tyler B. Smith[g], Tianling Zheng[h], Donald M. Anderson[a]

[a]Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

[b]South China Sea Environmental Monitoring Center, State Oceanic Administration, Guangzhou 510300, China

[c]Laboratoire des Microalgues Toxiques, Institut Louis Malardé, UMR 241-EIO, BP 30, 98713 Papeete Tahiti, French Polynesia

[d]Laboratory of Aquatic Environmental Science, Faculty of Agriculture, Kochi University, Otsu-200, Monobe, Nankoku, Kochi 783-8502, Japan

[e]Key Laboratory of Environment Change and Resources Use in Beibu Gulf, Ministry of Education, Guangxi Teachers Education University, Nanning 530001, China

[f]Coastal Watershed Institute, Florida Gulf Coast University, Fort Myers, Florida 33965, USA

[g]Center for Marine and Environmental Studies, University of the Virgin Islands, #2 John Brewers Bay, St Thomas, U.S. Virgin Islands 00802

[h]Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, School of Life Science, Xiamen University, Xiamen 361102, China

[§]These co-first authors contributed equally to this work

*Corresponding author: Mindy L. Richlen, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA; mrichlen@whoi.edu; Phone: 508-289-2552; Fax: 508-457-2027

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Abstract

Gambierdiscus is a genus of benthic dinoflagellates commonly associated with ciguatera fish poisoning (CFP), which is generally found in tropical or sub-tropical regions around the world. Morphologically similar species within the genus can vary in toxicity; however, species identifications are difficult or sometimes impossible using light microscopy. DNA sequencing of ribosomal RNA genes (rDNA) is thus often used to identify and describe *Gambierdiscus* species and ribotypes, but the expense and time can be prohibitive for routine culture screening and/or large-scale monitoring programs. This study describes a restriction fragment length polymorphism (RFLP) typing method based on analysis of the large subunit ribosomal RNA gene (rDNA) that can successfully identify at least nine of the described *Gambierdiscus* species and two *Fukuyoa* species. The software programs DNAMAN 6.0 and Restriction Enzyme Picker were used to identify a set of restriction enzymes (*Spe*I, *Hpy*CH4IV, and *Taq*αI) capable of distinguishing most of the known *Gambierdiscus* species for which DNA sequences were available. This assay was tested using *in silico* analysis and cultured isolates, and species identifications of isolates assigned by RFLP typing were confirmed by DNA sequencing. To verify the assay and assess intra-specific heterogeneity in RFLP patterns, identifications of 63 *Gambierdiscus* isolates comprising ten *Gambierdiscus* species, one ribotype, and two *Fukuyoa* species were confirmed using RFLP typing, and this method was subsequently employed in the routine identification of isolates collected from the Caribbean Sea. The RFLP assay presented

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1 RFLP assay design and in silico testing

For the assay design and *in silico* testing, sequences of the D1-D3 hypervariable region of the LSU rDNA from 12 *Gambierdiscus* species, one ribotype, and three *Fukuyoa* species were downloaded from the NCBI GenBank database, and the D1-D2 region was selected and used in subsequent analyses. Sequence data from this region was not available for *G. lapillus* and *G. balechii*, so these species were excluded from this analysis. The software programs DNAMAN 6.0 (Lynnon Biosoft, Quebec, Canada) and Restriction Enzyme Picker (Collin and Rocap, 2007) were used to identify restriction enzymes that could distinguish these species. DNAMAN ENZ (Enzyme file), which contains 2523 enzymes, was used in the *in silico* analysis. The fragments with all cutters and ends were considered. Restriction Enzyme Picker was then used to optimize the enzyme combination, according to the principle of minimum fragments and enzyme number. *Spe*I was selected to distinguish *G. australes* from *G. carolinianus*, *Taq*αI for *F. ruetzleri* and *F. yasumotoi*, and *Hpy*CH4IV for the remaining *Gambierdiscus* species.

2.2 Strain isolation and culture maintenance

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

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

2.3. RFLP analysis and sequencing

To verify the RFLP method, forty *Gambierdiscus* isolates, primarily from the Caribbean Sea, were identified using RFLP typing, and the species identity was confirmed using DNA sequencing. For these analyses, DNA was extracted from 1 ml of dense culture using the MoBIO PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions and eluted in a final volume of 100 µl. Partial fragments of the LSU rRNA gene were amplified from isolates using either the primers D1R and D2C (Scholin et al., 1994) or FD8 and RB (Chinain et al., 1999a). Each PCR reaction (25 µl) contained ~5 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 in an Eppendorf Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) with the following cycling conditions: 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 products were visualized by electrophoresis on 2% TAE agarose gel to verify that the PCR reaction was successful, and to assess the uncut PCR product size. PCR products used in the RFLP assay were then purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany).

RFLP reactions (25 µl) contained 4 µl of purified D1-D2 PCR product (variable, but generally ranging from ~100-200 ng), 18 µl water, 2.5 µl 1× CutSmart® Buffer, and 0.5 µl (5 U)

of *Spe*I and *Hpy*CH4IV (New England Biolabs, Inc., Ipswich, MA, USA). Samples were covered with plastic wrap to prevent evaporation and incubated at 37 °C for 15 min in the Eppendorf thermal cycler. The temperature was reduced to 4 °C and 0.5 µl (50 U) *Taq*αI (New England Biolabs, Inc.) was added to each reaction, then samples were incubated at 65 °C for 15 min followed by 80 °C for 20 min to deactivate the enzymes. RFLP digestion products, along with a 100 bp DNA ladder, were separated by electrophoresis on 2% TAE agarose gel at 75V for 1.5 hours.

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

3. **Results**

In silico analyses identified a combination of three enzymes (*Spe*I, *Hpy*CH4IV, and *Taq*αI) that produced fragments unique to the *Gambierdiscus* species examined, as well as two morphologically similar *Fukuyoa* species. *Spe*I distinguished *G. australes* and *G. carolinianus*, *Taq*αI distinguished *F. ruetzleri* and *F. yasumotoi*, and *Hpy*PYCH4IV differentiated among the remaining *Gambierdiscus* species. *Gambierdiscus toxicus* did not contain any restriction recognition sites, and generated a PCR product of ca. 726 bp. Enzyme recognition sites are listed in Table 2, and expected fragment sizes from the *in silico* analysis are provided in Table 3.

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

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

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

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

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

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

Discussion

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

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

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

In this study, the restriction enzymes *Spe*I, *Hpy*CH4IV, and *Taq*αI were selected for the assay based on successful *in silico* analysis, and this combination was tested using DNA extracts of ten *Gambierdiscus* species, one ribotype, and two morphologically similar *Fukuyoa* species.

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

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

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

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

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

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

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

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

Conclusions

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

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Tables

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

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

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

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

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

[a] Isolate from culture collection maintained by the Institut Louis Malardé, Tahiti, French Polynesia
[b] Isolate from culture collection maintained by the National Center for Marine Algae and Microbiota at Bigelow Laboratory, East Boothbay, ME, USA
[c] see Chinain et al. (2010)
[d] see Nishimura et al. (2013)
[e] see Chinain et al. (1999a)

Table 2. Recognition sites of the restriction enzymes *Spe*I, *Hpy*CH4IV, and *Taq*αI.

Enzyme	*Spe*I	*Hpy*CH4IV	*Taq*αI
Recognition site	A/CTAGT	A/CGT	T/CGA

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

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

* Smaller fragment size estimated from agarose gel.

Figures

Figure 1. RFLP profiles of LSU rDNA (D1-D2 hypervariable regions) from ten *Gambierdiscus* species, one ribotype, and two *Fukuyoa* species. Cari: *G. caribaeus* (BP Aug 08), Caro: *G. carolinianus* (GHCG2-C6), Beli: *G. belizeanus* (CCMP399), Paci: *G. pacificus* (Tub ET1), Ribo2: *Gambierdiscus* ribotype 2 (SH Dec 10-10), Yasu*: F. yasumotoi* (HGB), Carp: *G. carpenteri* (HGB6), Aust: *G. australes* (RAV-1), Poly: *G. polynesiensis* (Rai1), Toxi: *G. toxicus* (GTT-1), Ruez: *F. ruetzleri* (CCMP 3143), Silv: *G. silvae* (SH Apr 11-1), Scab: *G. scabrosus* (M080828_3), Mk: DNA marker.

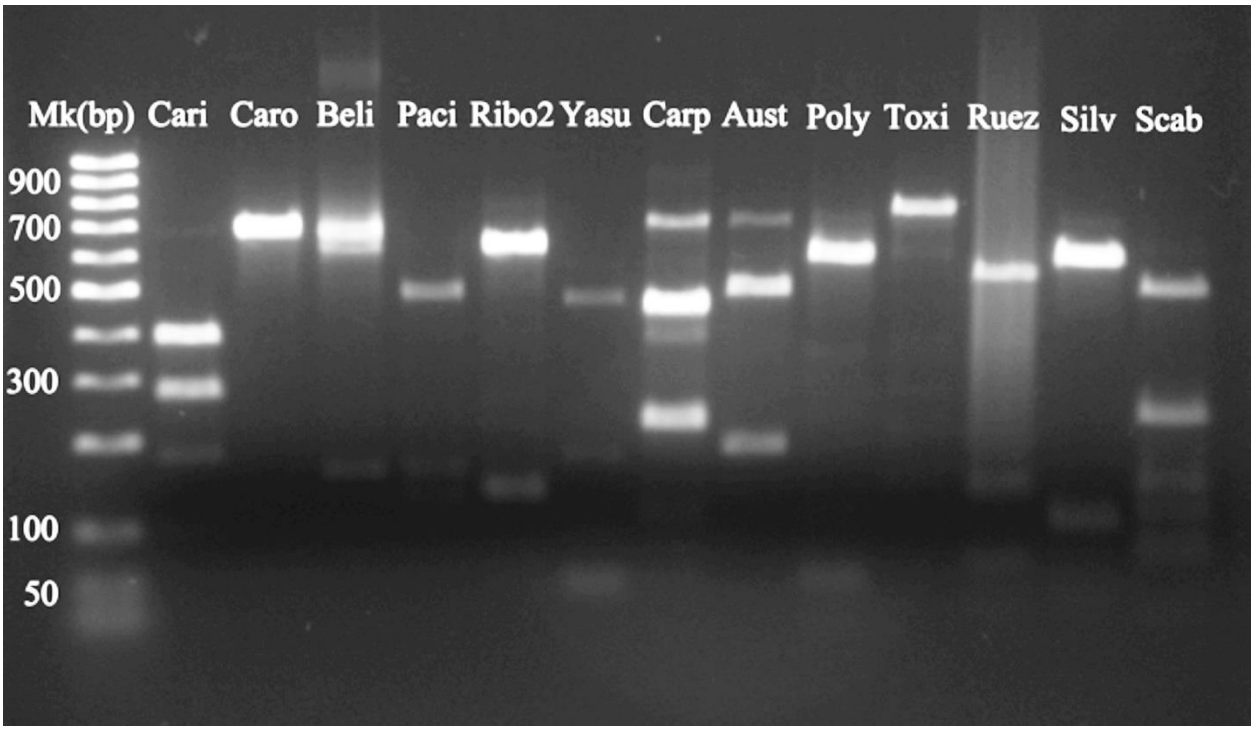
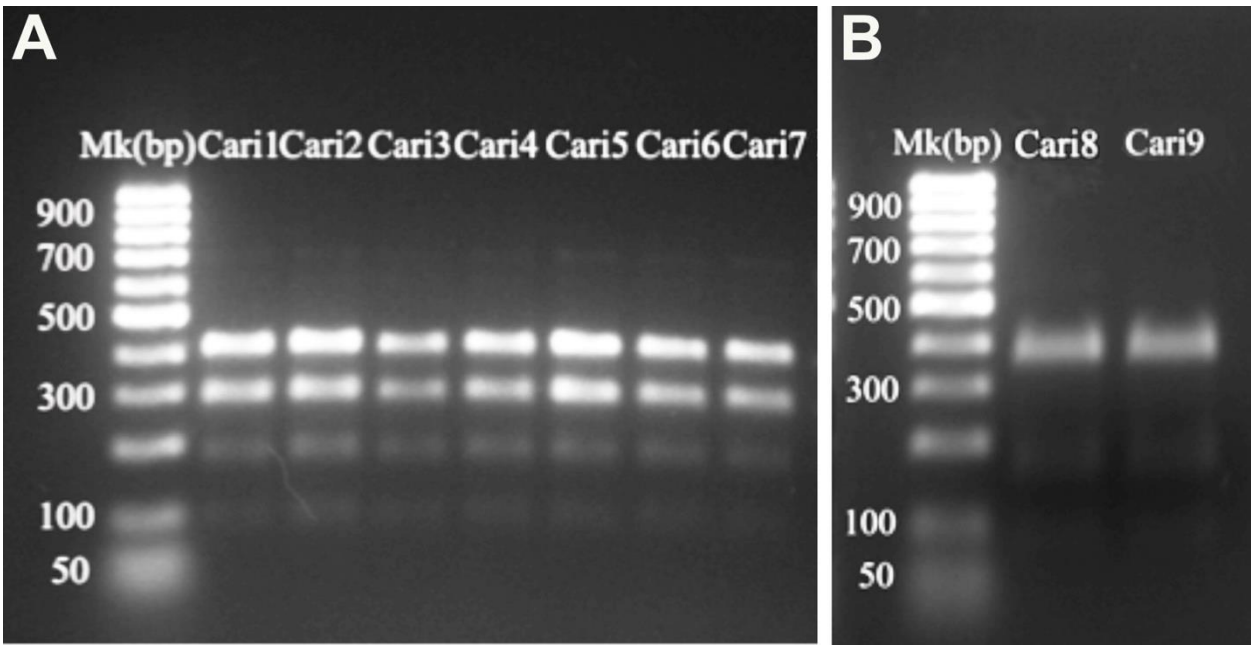
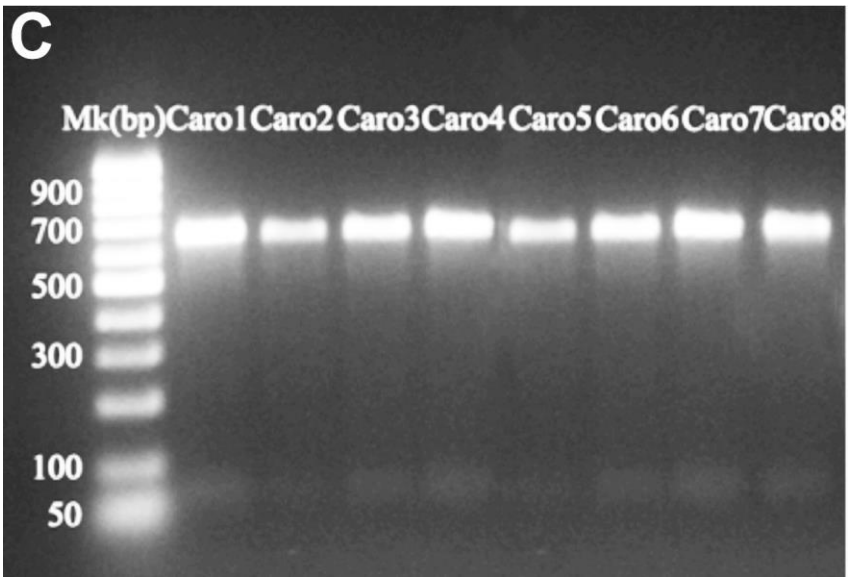
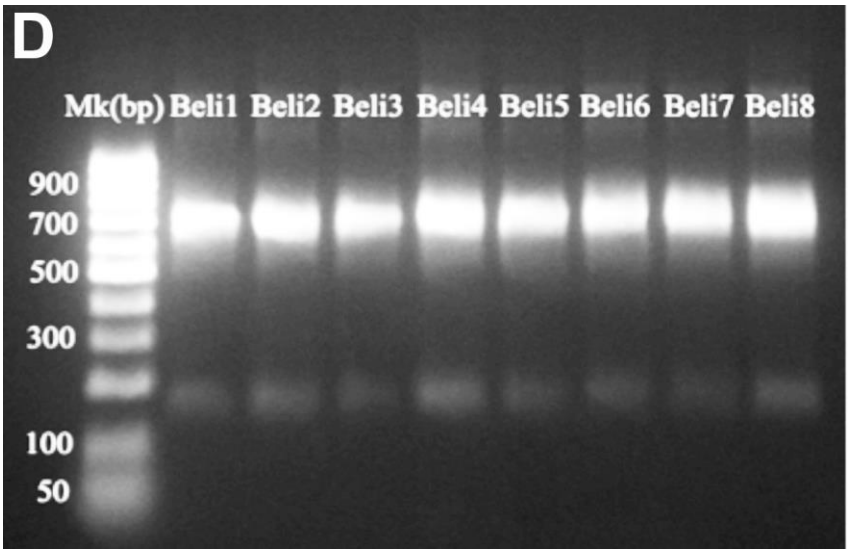
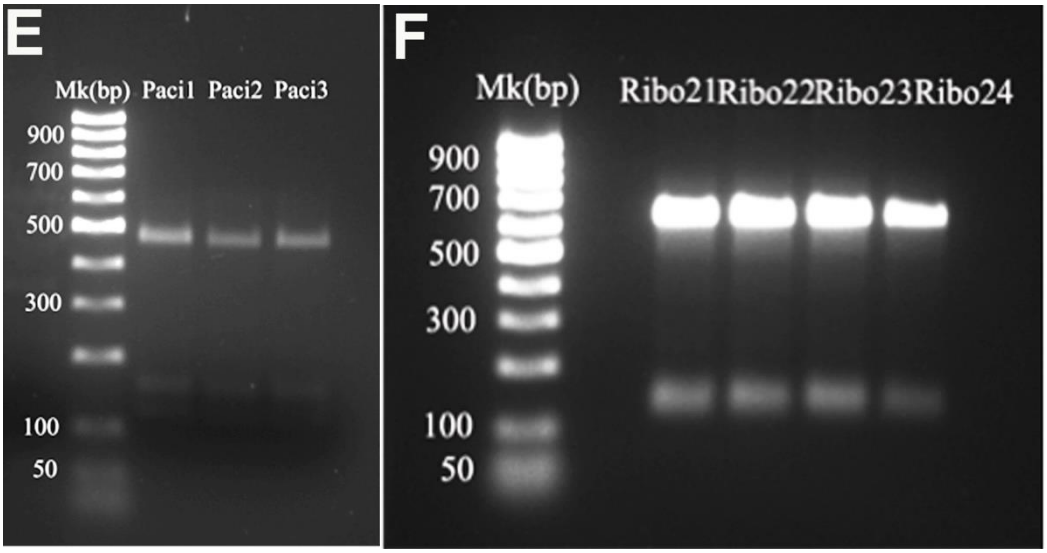


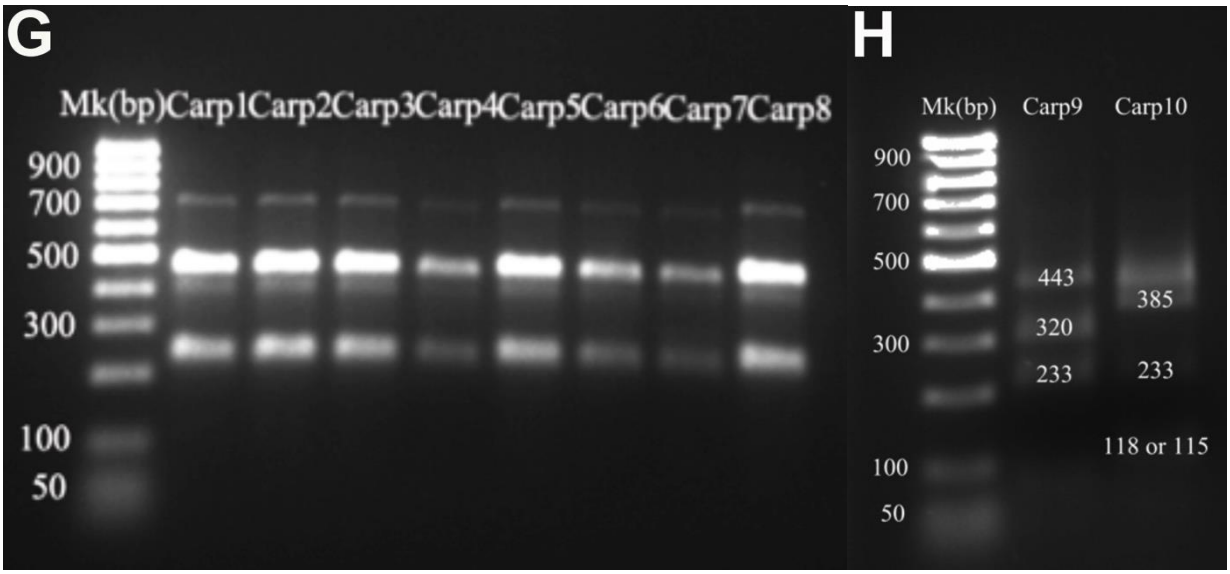
Figure 2. RFLP profiles of LSU rDNA (D1-D2 hypervariable regions) of conspecific strains. A: *G. caribaeus* from Caribbean Sea (Cari1~7), B: *G. caribaeus* from French Polynesia (Cari8~9), C: *G. carolinianus* (Caro1~8), D: *G. belizeanus* (Beli1~8), E: *G. pacificus* (Paci1~3), F: *Gambierdiscus* ribotype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

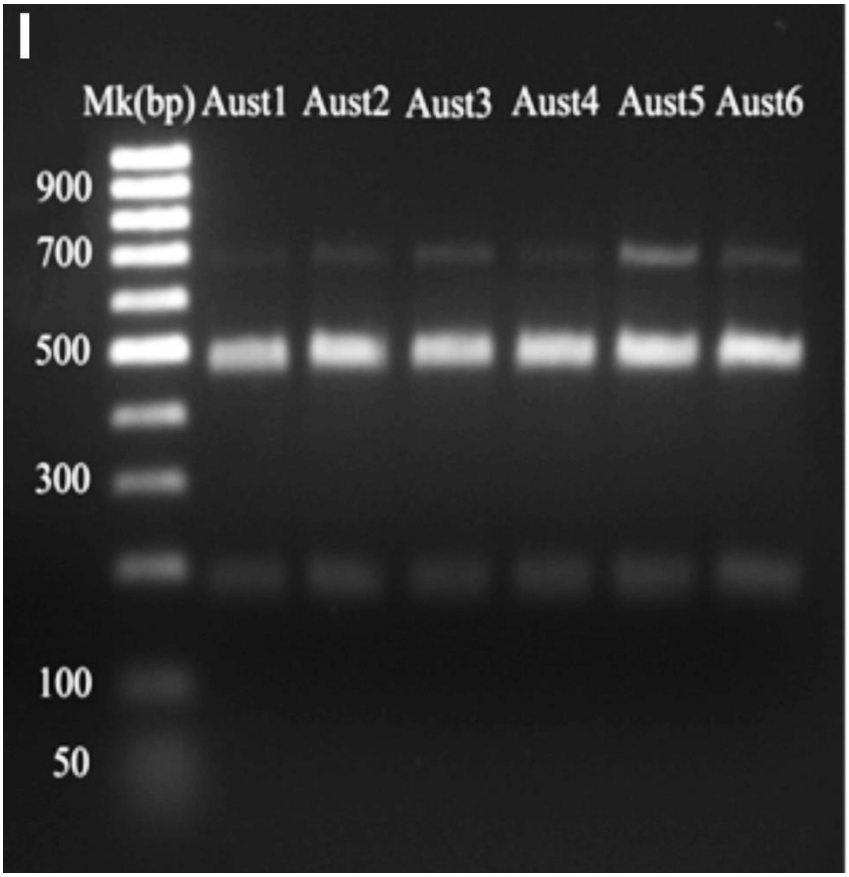



C
Mk(bp)
Caro1
Caro2
Caro3
Caro4
Caro5
Caro6
Caro7
Caro8
900
700
500
300
100
50


D
Mk(bp)
Beli1
Beli2
Beli3
Beli4
Beli5
Beli6
Beli7
Beli8
900
700
500
300
100
50

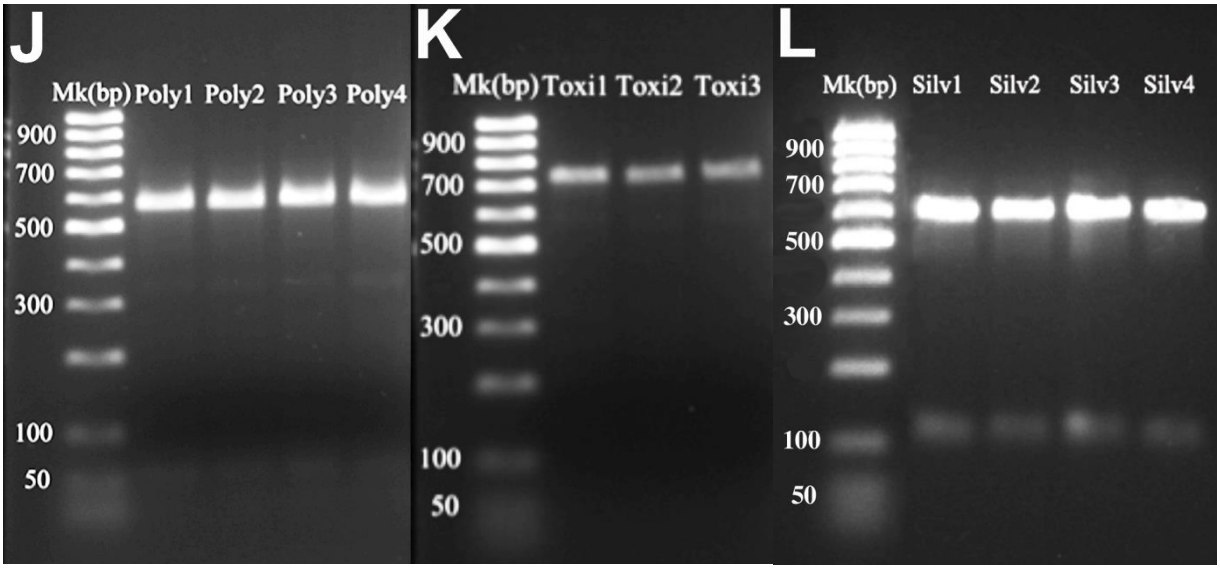

E
Mk(bp)
Paci1
Paci2
Paci3
900
700
500
300
100
50
F
Mk(bp)
Ribo21
Ribo22
Ribo23
Ribo24
900
700
500
300
100
50


G
Mk(bp)
Carp1
Carp2
Carp3
Carp4
Carp5
Carp6
Carp7
Carp8
900
700
500
300
100
50
H
Mk(bp)
Carp9
Carp10
900
700
500
300
100
50
443
320
233
385
233
118 or 115


I
Mk(bp)
Aust1
Aust2
Aust3
Aust4
Aust5
Aust6
900
700
500
300
100
50

1

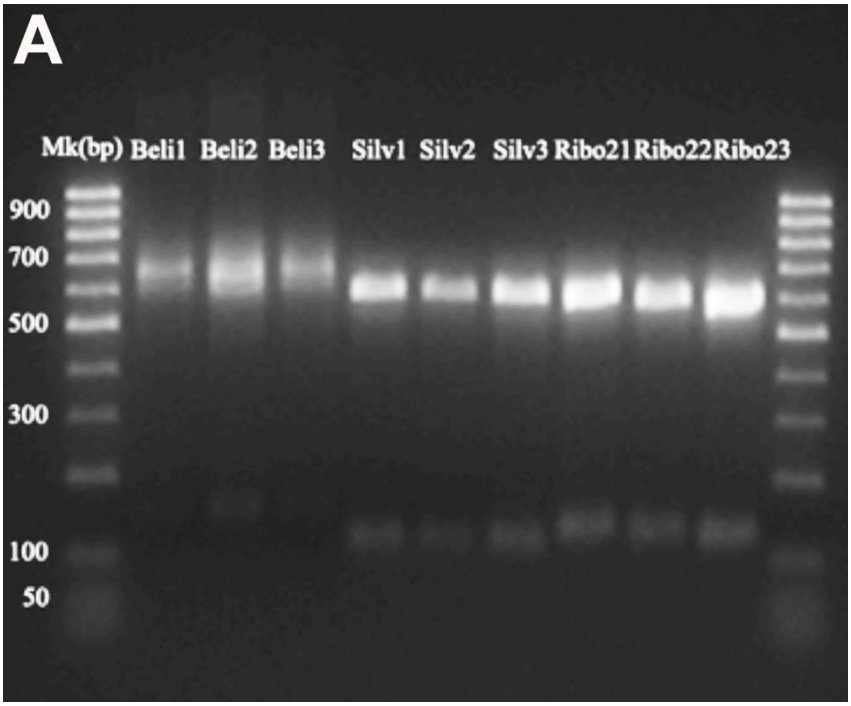
2

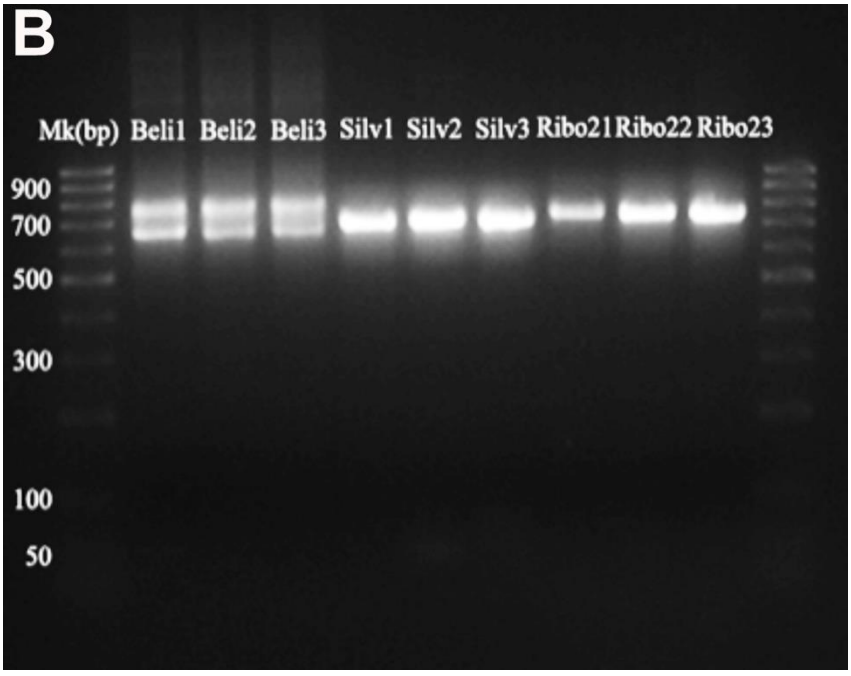

J
Mk(bp)
Poly1
Poly2
Poly3
Poly4
900
700
500
300
100
50
K
Mk(bp)
Toxi1
Toxi2
Toxi3
900
700
500
300
100
50
L
Mk(bp)
Silv1
Silv2
Silv3
Silv4
900
700
500
300
100
50

3

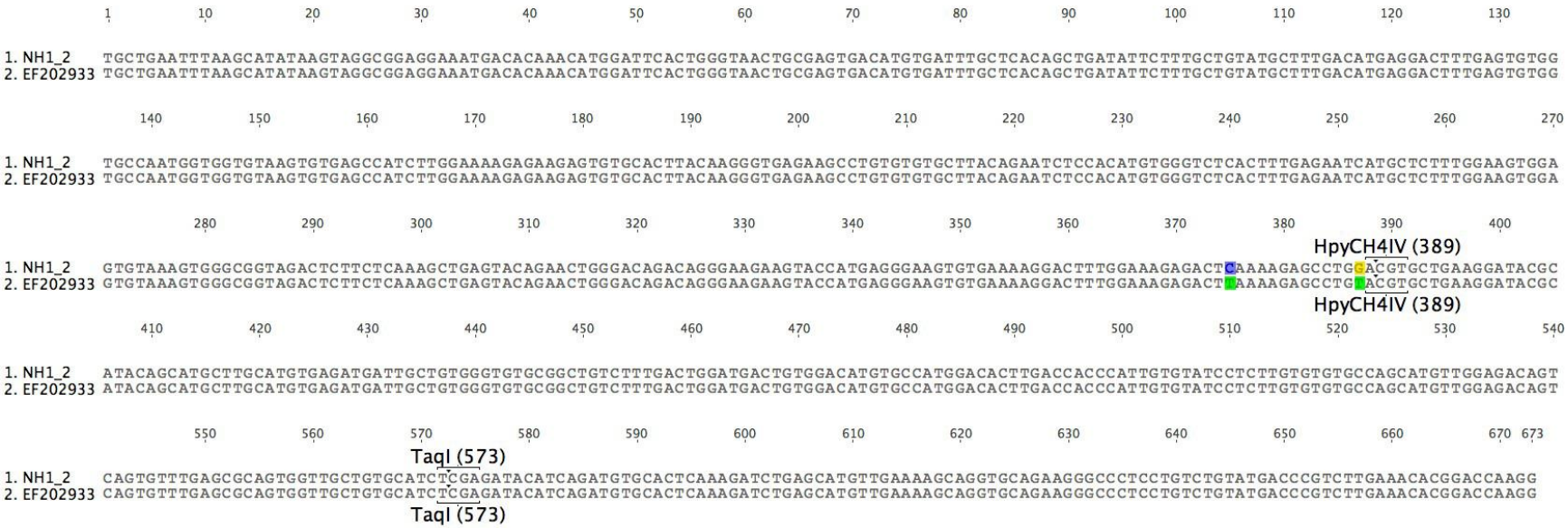
4

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

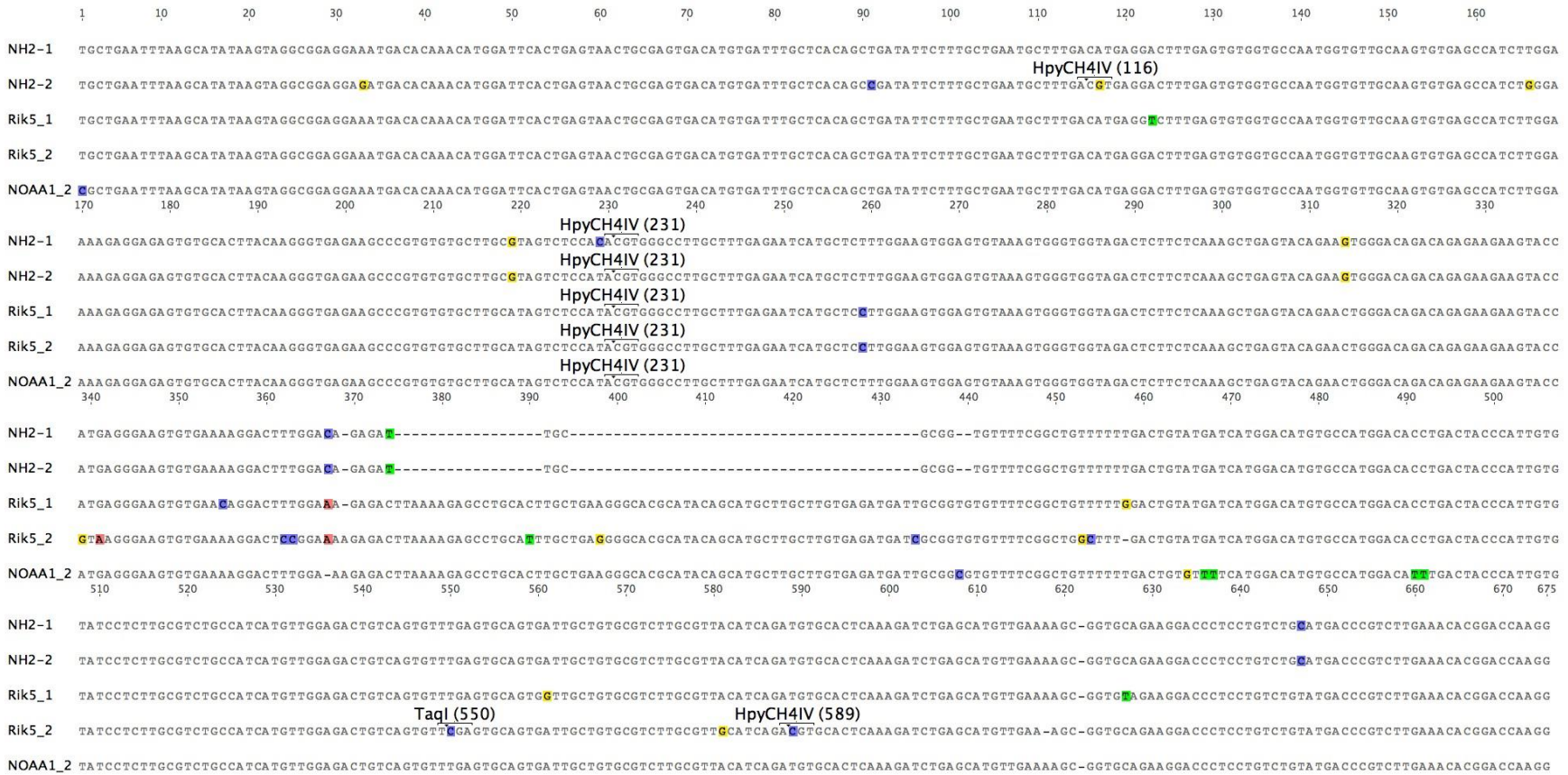




Supplementary Figure S1. Alignment of D1-D2 LSU rRNA gene sequence from *G. caribaeus* isolate NH-1 from French Polynesia, along with a *G. caribaeus* sequence from GenBank (accession no. EF202933), annotated with known restriction sites.



Supplementary Figure S2. Alignment of D1-D2 LSU rRNA gene sequences from *G. carpenteri* isolates from French Polynesia (NH-2 and Rik-5), along with a *G. carpenteri* sequence from GenBank (accession no. EF202938), annotated with known restriction sites.



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