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Development and Validation of PCR-RFLP Assay for Identification of *Gambierdiscus* species in the Greater Caribbean region

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Development and Validation of PCR-RFLP Assay for Identification of *Gambierdiscus* species in the Greater Caribbean region

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Abstract

Development and Validation of PCR-RFLP Assay for Identification of *Gambierdiscus* species in the Greater Caribbean region

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The genus *Gambierdiscus* is a recognized group of marine epiphytic-benthic dinoflagellates that produce the toxins that cause Ciguatera Fish Poisoning (CFP). To date, 12 species and 6 ribotypes of *Gambierdiscus* have been identified, and multiple species commonly co-occur within a single site or epiphyte community. Toxicity can vary by species, thus it is important to be able to differentiate between the species for research and monitoring purposes. *Gambierdiscus* species have very similar morphological characteristics and are very difficult or impossible to distinguish using light microscopy. DNA sequencing has been an important tool in the definition of the *Gambierdiscus* species, but it can be time-consuming and relatively expensive. To provide an alternative approach, I developed a PCR-RFLP protocol for efficient, rapid, and cost-effective identification of *Gambierdiscus* species in the Gulf of Mexico and Caribbean Sea, where CFP cases and *Gambierdiscus* spp. have been reported. The assay targets the D1-D2 hypervariable regions of the large subunit ribosomal RNA gene and uses a single restriction enzyme (BsrI). This method produces distinct RFLP banding patterns for the six species of *Gambierdiscus* reported from the Gulf of Mexico and Caribbean Sea, and it distinguishes them from the four Pacific endemic species. This method was successfully used to type 496 clonal isolates of *Gambierdiscus* from the U.S. Virgin Islands and expands the tools available to researchers and managers engaged in monitoring activities and ecological studies.

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Introduction

The genus *Gambierdiscus* (Adachi and Fukuyo 1979) is a recognized group of marine epiphytic-benthic dinoflagellates that produce gambiertoxins, a precursor of the ciguatoxins that cause Ciguatera Fish Poisoning (CFP). Globally, CFP is the most common foodborne illness associated with consumption of subtropical and tropical marine finfish (Yasumoto *et al*. 1977, Friedman *et al*. 2008). Macroalgae, where *Gambierdiscus* cells, which dwell on the surface of macroalgae, are grazed by herbivorous fish species, which are then eaten by carnivorous fishes, resulting in the bioconversion of gambiertoxins to the more potent ciguatoxins within the food web (Heimann and Sparrow 2015). Also, studies have identified distinct pathways in the food chain transfer of gambiertoxins and ciguatoxins (Kelly *et al*. 1992, Lewis and Holmes 1993). *Gambierdiscus toxicus* Adachi et Fukuyo, the first species of this genus, was described in 1979 from samples taken around Tahiti and the Gambier Islands (South Pacific Ocean) where CFP frequently occurs (Adachi and Fukuyo 1979). Yasumoto *et al*. (1977) identified this dinoflagellate as the causative agent of CFP based on the correlation between the amount of the toxins and the number of dinoflagellate cells.

CFP causes significant public health and economic impacts and is expanding to non-endemic regions worldwide (Dickey and Plakas, 2010, Heimann and Sparrow 2015). Humans become sick (gastrointestinal, neurological and cardiovascular symptoms) after consuming fish contaminated with ciguatoxins (Lehane and Lewis 2000). More than 400 commercially important fish species have been associated with ciguatoxins, including top predators like barracuda, grouper, moray eel, snapper and in some cases, small herbivorous fish (Halstead 1978, Lehane and Lewis 2000, Van Dolah 2000, Caillaud *et al*. 2010, Chan *et al*. 2011). CFP has enormous economic implications, mainly to local fisheries in developing countries (Lehane and Lewis 2000). In the U.S., Anderson *et al.* (2000) estimate that CFP costs on average $21 million per year, primarily due to health care costs and lost productivity. Annually, there is an estimate of 50,000 – 500,000 cases of CFP around of the world; estimates vary widely because CFP is vastly underreported, especially in ciguatera-endemic areas where residents know there is no effective treatment (Fleming *et al*. 1998). Puerto Rico and the U.S. Virgin Islands alone have been reported 20,000 – 40,000 cases of CFP per year (Tosteson 1995). CFP is limited to tropical and subtropical areas (mostly in the Pacific and Indian Ocean, and Caribbean Sea) but in recent years it has been reported from new areas, especially due to tourism and trade of seafood from endemic areas (Dickey and Plakas 2010, Vandersea *et al*. 2012). Also, climate change and anthropogenic activities are factors that influence the development of CFP events (Moore *et al*. 2008, Parsons *et al*. 2012). Thus, both the incidence and worldwide distribution of CFP appear to be increasing, which represent a public health and economic threat in the future.

As CFP is related to the presence of *Gambierdiscus* spp. (Yasumoto *et al*.1977, Bagnis *et al*. 1980), the study of this marine dinoflagellate is important to understand and predict CFP risk. Studies have determined that toxicity and production of ciguatoxins can differ amongst species or strains, therefore the relationship between *Gambierdiscus* spp. and ciguatera is likely more complex than first thought (Chinain *et al*. 1999, Lewis 2006, Chinain *et al*. 2010). For many years *G. toxicus* was considered the only species in this genus, but in the last two decades more species have been identified in different sites around the world (Appendix A). Initially, five new species found in Belize, Singapore and French Polynesia were identified, based on their cell morphology, thecal plate characteristics or molecular analysis: *G. belizeanus* M. A. Faust, *G. yasumotoi* M. J. Holmes, *G. pacificus* Chinain et Faust, *G. australes* Faust et Chinain, and *G. polynesiensis* Chinain et Faust (Faust 1995, Holmes 1998, Chinain *et al*. 1999). A more recent and extensive study identified four more species as *G. caribaeus* Vandersea, Litaker, Faust, Kibler, Holland and Tester, *G. carolinianus* Litaker, Vandersea, Faust, Kibler, Holland et Tester, *G. carpenteri* Kibler, Litaker, Faust, Holland, Vandersea, et Tester, and *G. ruetzleri* Faust, Litaker, Vandersea, Kibler, Holland et Tester, and determined the phylogenetic relationship of the ten species described until that time (Litaker *et al*. 2009). The molecular analysis gave support to the morphological separation of these 10 species of *Gambierdiscus* and showed that it is a monophyletic group. Lately, new species from Canary Islands in Spain and coastal waters in Indonesia were described as *G. excentricus* S. Fraga (Fraga *et al*. 2011) and *G. balechii* S. Fraga, F. Rodríguez et I. Bravo (Fraga *et al*. 2016). From these species described, *G. yasumotoi* and *G. ruetzleri* showed enough morphological (globular form) and molecular differences (SSU and LSU phylogenies) to reclassify them in a new genus called *Fukuyoa* (Gomez *et al*. 2015). Also, phylogenetic analysis has described eight phylotypes. Litaker *et al*. (2010) identified *G*. sp. ribotype 1, and *G.* sp. ribotype 2 in the Caribbean Sea. Kuno *et al*. (2010) and Nishimura *et al*. (2013) identified *G.* sp. type 1, *G.* sp. type 2 and *G.* sp. type 3 from Japanese coastal waters. Xu *et al*. (2014) identified *G.* sp. type 4, *G.* sp. type 5 and *G.* sp. type 6 in a small atoll located in the central Pacific Ocean.

Currently, *G*. sp. ribotype 1 and *G*. sp. type 1 are known to be synonymous with *G. silvae* S. Fraga et F. Rodríguez (Fraga and Rodríguez 2014) and *G. scabrosus* T. Nishim., Shin. Sato et M. Adachi (Nishimura *et al*. 2014), respectively. Therefore, to date there are 12 species and six ribotypes of *Gambierdiscus* described using both morphological characteristics (anterio-posteriorly compressed cell with lenticular shape) and molecular tools.

The various *Gambierdiscus* species appear to be geographically restricted to particular ocean basins, based on the areas from which cultured representatives have been found (Litaker *et al*. 2009). For example, several *Gambierdiscus* species were isolated only from the Atlantic Ocean, whereas others were isolated only from the Pacific Ocean; others like *G. carpenteri* and *G. caribaeus* were found in both oceans. This study shows that the genus *Gambierdiscus* is widely distributed, but that many species may be endemic to particular regions. Apparently in recent years, the latitudinal distribution of *Gambierdiscus* species has been increasing from tropical to temperate areas around the world due to effects of climate change (Moore *et al*. 2008). Gulf of Mexico and the U.S. Atlantic coast are areas where CFP cases and *Gambierdiscus* spp. were reported recently, indicating the risk of ciguatera in the region (Villareal *et al*. 2007, CDC 2009). The Caribbean Sea is an endemic area of *Gambierdiscus* spp., so it could be the source of this microalgae into the Gulf of Mexico through the marine current system (Molinari *et al*. 1981). Except for these reports, there is little research on the different species of *Gambierdiscus* and their distribution in the Greater Caribbean Region (GCR). This is because a considerable amount of research was done in the 1980s, but all of it referred to *Gambierdiscus toxicus*, the only described species at that time. Studies in the Caribbean Sea and Gulf of Mexico have found six species of *Gambierdiscus* in a few areas (Chinain *et al*. 1999, Litaker *et al*. 2009, Litaker *et al*. 2010), thus more work is necessary at temporal and spatial level to characterize the diversity and ecology of this dinoflagellate this region. The presence of *Gambierdiscus* species represents a risk of CFP events in this region where fishing and tourism activities play an important role in the economy, highlighting the importance of study this dinoflagellate.

Molecular methods have been increasingly used for identification of *Gambierdiscus* species because morphological characteristics alone are not practical for routine identification. *Gambierdiscus* is described as unicellular, photosynthetic, benthic, and armored species with an antero-posteriorly compressed body shape, circular narrow deep cingulum, deep hollow sulcus and a theca with 33 plates (Adachi and Fukuyo 1979). These characteristics are very similar among species and can change with environmental factors, geographical location, or with the different stages of the life cycle, making it very difficult to distinguish one species from another using light microscopy. For example, in *Gambierdiscus* spp. the sulcal plate tabulations are difficult to identify with microscopy, and the apical pore plate shape varies over a wide range (Litaker *et al*. 2009). Also, the identification of species based on morphological features is time-consuming and requires significant taxonomic expertise. Molecular tools have the potential for easier and faster identification of species (Chinain *et al*. 1999, Litaker *et al*. 2009), as long as there is a molecular marker that is species-specific. Various studies have used ribosomal RNA (rRNA) gene sequences to identify species and determine their phylogenetic relationships and geographic distribution. *Gambierdiscus* phylogenetic studies have shown that it is a group with high genetic variability in the rRNA genes at intra- and interspecies level.

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a simple molecular method that can identify species or ribotypes. Although it has become less common due to the development of high throughput sequencing, it continues to be a very useful method for rapid screening of DNA sequence variation. It exploits the ability of restriction enzymes to cleave DNA at specific sequence recognition sites. With careful choice of the target DNA region and restriction enzyme, the result is a pattern of fragments with specific lengths (RFLP profile) that is different for each species and can be visualized by agarose gel electrophoresis. Open source bioinformatics tools have improved the identification of DNA sequences and restriction enzymes to use in the PCR-RFLP method. Online software programs use DNA sequences to perform virtual restriction enzyme digests and determine the best enzymes to detect variation at a specified level (species, strain, among others). Therefore, PCR-RFLP could provide a reliable, cheap, and rapid method to identify *Gambierdiscus* species in the GCR.

There is prior information that supports the use of specific DNA regions to identify species in the *Gambierdiscus* genus. Small (SSU) and large (LSU) subunit rRNA gene sequences have shown their value as molecular markers in the identification of the *Gambierdiscus* species (Chinain *et al.* 1999, Litaker *et al*. 2009). The genetic distances for close *Gambierdiscus* species based on the D1-D3 and D8-D10 regions show that the LSU rRNA provides good resolution to discriminate between very closely related species, with the D1-D3 region exhibiting higher genetic distances (Chinain *et al*. 1999, Litaker *et al*.

2009, Fraga *et al*. 2014). The D1-D2 hypervariable regions of the LSU rRNA gene, which is contained in the LSU rRNA D1-D3 region, show sufficient variability to differentiate between closely related species, making it a strong taxonomic marker for a wide variety of metazoa and protists (Sonnenberg *et al*. 2007, Wylezich *et al*. 2010, Santoferrara *et al*. 2013). This region is a useful genetic marker for the taxonomy of *Alexandrium* spp. dinoflagellates, and in some ecological studies it has been able to discriminate species and strains (Ki and Han 2007, Band-Schmidt *et al*. 2003). This information gives support to the possible use of the LSU rRNA D1-D2 region as a genetic marker to discriminate related species in *Gambierdiscus* genus.

The goal of this study was to develop a PCR-RFLP assay using the LSU rRNA D1-D2 hypervariable regions as a molecular marker to distinguish among species of the dinoflagellate *Gambierdiscus* found in the GCR. Many studies have identified *Gambierdiscus* species as *Gambierdiscus* spp. or *G. toxicus*, but new studies have increased the number of known species. Therefore, many of those previous works could actually be referring to other named species. Consequently, a method for a fast and reliable identification of *Gambierdiscus* species is needed, and very important for future ecologic studies and monitoring activities. This study is part of a project investigating *Gambierdiscus* ecology in the GCR. The sampling sites are located in the US Virgin Islands, an area that is known to have both CFP and *Gambierdiscus* presence. In this study, existing LSU rRNA sequences of *Gambierdiscus* spp. were used to design a PCR-RFLP method that could discriminate the *Gambierdiscus* species. This assay was validated using a set of independently identified DNA samples from different strains of 11 *Gambierdiscus* species. Lastly, it was used to type a collection of *Gambierdiscus* strains isolated from the US Virgin Island in order to evaluate the utility of this PCR-RFLP method for identifying the different species present in the island. The information obtained from this area can be applied across the GCR.

Materials and methods

SINGLE CELL ISOLATION TO ESTABLISH CULTURED STRAINS

Dinoflagellates samples were collected from U.S. Virgin Islands in the Caribbean Sea (Figure 1). Sampling sites were located in the south area of St. Thomas Island: Black Point (BP), Flat Cay (FC), Benner Bay (BB) and Seahorse (SH). Water and macroalgae samples were taken monthly from August 2013 to July 2015 in these four sites. Macroalgae were picked by hand by SCUBA divers and placed into a one gallon plastic zipper bag with a small amount of surrounding seawater. This was continued until the bag was almost full with samples of multiple macroalgal species. The bag was stored in a cooler and returned to the lab. There, the bag was agitated to displace the epiphyte cells from the macroalgae. The seawater from the bag was sequentially filtered through 200µm and 20µm nylon mesh sieves. The material retained on the 20µm sieve was rinsed with filtered seawater into a beaker and then transferred into plastic tissue culture flasks for overnight shipment to the Marine Science Institute at Port Aransas (Texas).

In the laboratory, 1 ml of sterile modified K Medium was added to each container from the field (Keller *et al*. 1987). Cultures of *Gambierdiscus* strains from single isolated cells were established for DNA extraction. Every cell was cultured in the laboratory in modified K medium (Keller *et al*. 1987) prepared with 0.2 µm-filtered and autoclaved natural seawater. Cells were grown in borosilicate culture tubes at 27 ^{o}C under 12:12 light:dark conditions with 90 µmol photons m^{-2} s^{-1} irradiance. From every sample, I isolated at least 12 single cells via microcapillary tubes using a stereo microscope. Using a petri dish, every individual dinoflagellate cell isolated was sequentially transferred through five or more drops of sterile modified K medium to remove contaminants. Each cell was then transferred to a single well of a 96 well cell culture plate containing 200 µl of modified

K medium. After successful growth of more than 8 cells (8 - 10 days), all of the cells from a well were transferred to a 15 ml tube with 5 ml of medium. After 10 – 15 days, 1 ml of culture from the 15 ml tube was transferred to a 55 ml tube containing 20 ml of medium. *Gambierdiscus* cells were grown for ~25 days in this tube, and then 1 ml of culture was transferred to a new tube. This second 20 mL transfer was done in duplicate, one tube was kept to maintain the strain in the laboratory and a replicate tube was used for DNA extraction when sufficient cell density was achieved (~25 days). Morphological identification was done for each isolate using light microscopy to determine if it belongs to the *Gambierdiscus* genus.

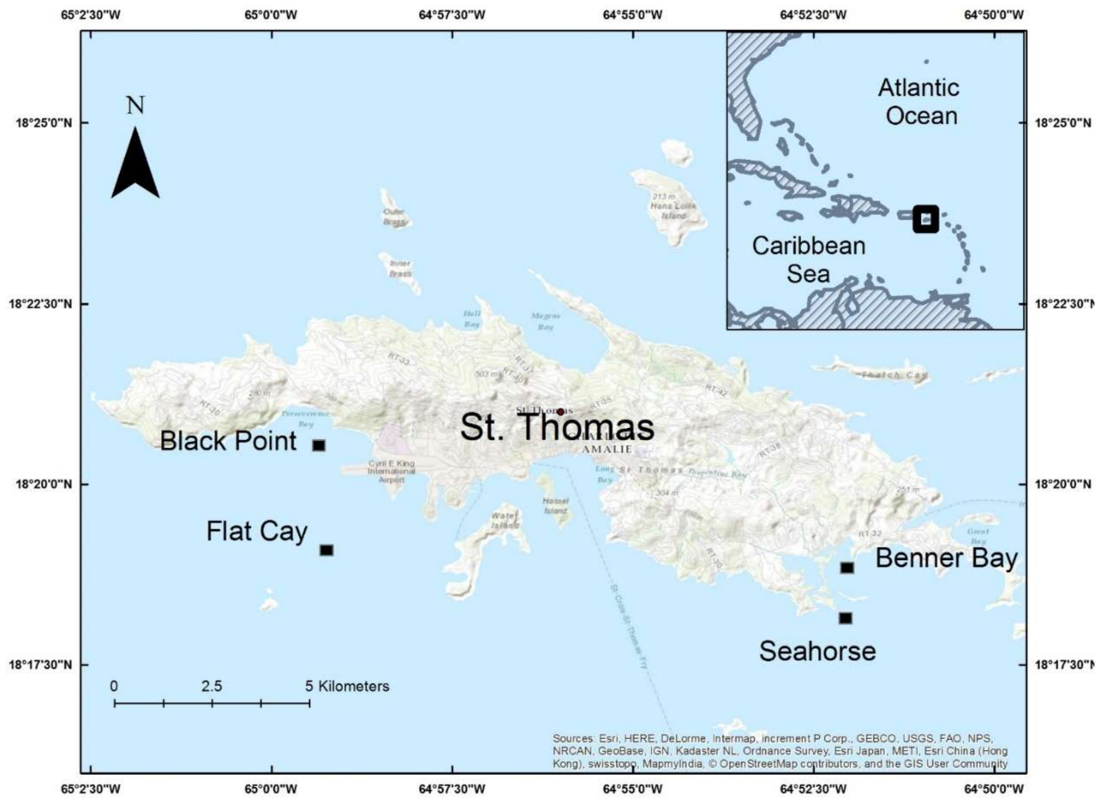

Figure 1. Study area with localization of the points of sampling in St. Thomas - US Virgin Islands in the Caribbean Sea.

DNA EXTRACTION FROM CULTURES

In almost all the tubes, the cells were found in the bottom of the tubes, but in some tubes the cells were found floating along the tube surrounded by a polymer which apparently increases their buoyancy. Cultures in exponential phase were harvested by taking cells from the bottom or water column of the culture tube using a micropipette. Typical *Gambierdiscus* cell density in the cultures was ~700 cells/ml. The cells were transferred to a 1.5 ml microcentrifuge tube and collected by centrifugation for 3 minutes at 3000 x g. The cells were washed twice with PBS buffer (BupH™ Phosphate Buffered Saline Pack – Thermo Fisher Scientific Inc) by re-suspending in 1.5 ml of buffer followed by centrifugation, and the supernatant discarded. This process improved the DNA extraction from the *Gambierdiscus* cells by removing contaminants and enzyme inhibitors. DNA was extracted from the resulting cell pellets using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). The protocol was modified by the addition of a quarter volume of 0.5 mm silica-zirconium beads (BioSpec Products, Inc. Bartlesville, OK, USA) to the 180 µl Buffer ATL in the first step, followed by 1 minute of vortex mixing at maximum speed (Erdner *et al*. 2011). Whole genomic DNA was eluted twice with 100 µl Buffer AE with a final elution volume of 200 µL and stored at -20 ^{o}C. The DNA preparations were quantified using a NanoVue Plus spectrophotometer (GE Healthcare, UK). DNA concentration ranged from 0.2 to 125.5 µg/ml.

RFLP ASSAY DESIGN

The online open source software RestrictionMapper (http://www.restrictionmapper.org) was used to find candidate enzymes that would provide species-specific discrimination of *Gambierdiscus* isolates based on their LSU rRNA D1-D2 sequences. This program finds restriction endonuclease cleavage sites in DNA sequences and performs virtual RFLP with a database of restriction enzymes to determine the enzyme(s) that will distinguish the input sequences. As an input file, I used LSU rRNA D1-D2 sequences from one strain of each species, provided by M. Richlen at the Woods Hole Oceanographic Institution. RestrictionMapper results indicated that the BsrI restriction enzyme could distinguish the six *Gambierdiscus* species found in the GCR, according to the criteria of minimizing fragments and enzyme number.

PCR AMPLIFICATION OF LSU RRNA

The D1-D2 region of the hypervariable region of the large subunit (LSU) rRNA was amplified using the primers D1R (5'-ACCGCTGAATTTAAGCATA-3') and D2C (5'-CCTTGGTCCGTGTTTCAAGA-3') (Scholin *et al*. 1994). PCR amplification reactions (25 µl) contained ~5 ng template DNA, 1X PCR Buffer (500 mM KCL and 100 mM Tris-HCl, pH 8.3), 0.25 mM of each dNTP, 0.5 µM of D1R primer, 0.5 µM of D2C primer, and 0.625 U of Taq DNA Polymerase (Takara Taq Bio Inc). Hot start PCR amplification was performed using a Eppendorf Mastercycler thermocycler following these conditions: 5 minutes denaturing at 94 ^{o}C (after 1-2 minutes at 94 ^{o}C it was paused to add the Taq), followed by 35 cycles of 30 seconds denaturing at 94 ^{o}C, 1 minute annealing at 50 ^{o}C, 2 minutes elongation at 72 ^{o}C, and a final elongation for 10 minutes at 72 ^{o}C. Successful amplification was verified using 3 µL of each PCR reaction mixed with 2µL of loading dye containing GelRedTM (300x dilution) nucleic acid gel stain (Biotium, Hayward, CA, USA), checked by 0.8% agarose gel electrophoresis (0.5X TBE) and visualized under UV light.

RFLP ANALYSIS

The BsrI restriction enzyme was used following manufacturer's recommendations (New England BioLabs, Beverly, MA, USA). Each restriction digest contained 2.5 µl NE-Buffer 3.1 (1X), 18 ul water, 0.3 µl of BsrI restriction enzyme, and 4µl of PCR product. Each sample was digested at 37℃ for 15minutes, followed by incubation at 65℃ for 30 minutes and inactivation at 80℃ for 20 minutes. From each digested sample, 6 µl of digest were mixed with 2 µl of loading dye with GelRedTM (300x dilution) nucleic acid gel stain (Biotium, Hayward, CA, USA) and loaded in the gel. The restriction products were resolved on a 2.0% agarose gel with 0.5X TBE buffer. Gel images of the RFLP band patterns were recorded using a UV camera (FOTO/Analyst® Express Systems, Foto/UV 26, Fotodyne Inc. Hartland, WI, USA).

The *in silico* assay was first tested using a panel of DNA extracts from *Gambierdiscus* species that had been positively identified by DNA sequencing of the D1-D2 region. These samples correspond to eleven different species and strains of *Gambierdiscus* and were provided by M. Richlen from the Woods Hole Oceanographic Institution (Table 1). Genomic DNA from some species and strains was scarce, therefore in some cases D1-D2 PCR products were used. Also, it was not possible obtain genomic DNA from *G. excentricus*, and *G*. sp. type 2 – 6.

To evaluate the applicability of this method using samples from the field, 496 DNA samples obtained from strains cultured in the laboratory were analyzed The details of these samples obtained from the U.S. Virgin Islands is presented in Appendix B.

Table 1. Strains of *Gambierdiscus* spp. used for assay validation, and results of RFLP analysis.

Isolates	Geographic Origin	Abbreviation	Molecular species ID	RFLP ID
BB Apr 11-11	St. Thomas, USVI	Cari1	*G. caribaeus*	*G. caribaeus*
BP Aug 08	St. Thomas, USVI	Cari2	*G. caribaeus*	*G. caribaeus*
HGB7	Florida Keys, FL, USA	Cari3	*G. caribaeus*	*G. caribaeus*
LKH4	Florida Keys, FL, USA	Cari4	*G. caribaeus*	*G. caribaeus*
Tenn10	Florida Keys, FL, USA	Cari5	*G. caribaeus*	*G. caribaeus*
BB may 10-12	St. Thomas, USVI	Cari6	*G. caribaeus*	*G. caribaeus*
1401BP2	St. Thomas, USVI	Cari7	No sequenced	*G. caribaeus*
1309FC4-7	St. Thomas, USVI	Cari8	No sequenced	*G. caribaeus*
GHCG2-C6	San Salvador, Bahamas	Caro1	*G. carolinianus*	*G. carolinianus*
GHCG2-A6	San Salvador, Bahamas	Caro2	*G. carolinianus*	*G. carolinianus*
GHCG2-B8	San Salvador, Bahamas	Caro3	*G. carolinianus*	*G. carolinianus*
Cheeca1	Florida Keys, FL, USA	Caro4	*G. carolinianus*	*G. carolinianus*
CCMP399	St. Barthelemy Island	Beli1	*G. belizeanus*	*G. belizeanus*
FC Dec 10-13	St. Thomas, USVI	Beli2	*G. belizeanus*	*G. belizeanus*
BP Apr 11-7	St. Thomas, USVI	Beli3	*G. belizeanus*	*G. belizeanus*
BP Mar 10-18	St. Thomas, USVI	Beli4	*G. belizeanus*	*G. belizeanus*
BP Mar 10-25	St. Thomas, USVI	Beli5	*G. belizeanus*	*G. belizeanus*
BP Mar 10-31	St. Thomas, USVI	Beli6	*G. belizeanus*	*G. belizeanus*
BP Mar 10-7	St. Thomas, USVI	Beli7	*G. belizeanus*	*G. belizeanus*
MUR4	Moruroa, French Polynesia	Paci1	*G. pacificus*	*G. pacificus*
HO91	Otepa, Hao, French Polynesia	Paci2	*G. pacificus*	*G. pacificus*
TubET1	Mahu, Tubuai, French Polynesia	Paci3	*G. pacificus*	*G. pacificus*
BP Apr 11-6	St. Thomas, USVI	Ribo2-1	*G.* sp. ribotype 2	*G.* sp. ribotype 2
SH Dec 10-10	St. Thomas, USVI	Ribo2-2	*G.* sp. ribotype 2	*G.* sp. ribotype 2
SH Dec 10-12	St. Thomas, USVI	Ribo2-3	*G.* sp. ribotype 2	*G.* sp. ribotype 2

Table 1: (continued)

TRL29	Florida Keys, FL, USA	Ribo2-4	*G.* sp. ribotype 2	*G.* sp. ribotype 2
KML1	Florida Keys, FL, USA	Carp1	*G. carpenteri*	*G. carpenteri*
TPH12	Florida Keys, FL, USA	Carp2	*G. carpenteri*	*G. carpenteri*
1506BB3	St. Thomas, USVI	Carp3	No sequenced	*G. carpenteri*
1402FC8	St. Thomas, USVI	Carp4	No sequenced	*G. carpenteri*
PO		Aust1	*G. australes*	*G. australes*
RAV1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Aust2	*G. australes*	*G. australes*
G3/93		Aust3	*G. australes*	*G. australes*
S080911-1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Aust4	*G. australes*	*G. australes*
ISC5G		Aust5	*G. australes*	*G. australes*
I080606-1		Aust6	*G. australes*	*G. australes*
Rai1		Poly1	*G. polynesiensis*	*G. polynesiensis*
Rik8		Poly2	*G. polynesiensis*	*G. polynesiensis*
RG92		Poly3	*G. polynesiensis*	*G. polynesiensis*
TB-92	Tubuai, French Polynesia	Poly4	*G. polynesiensis*	*G. polynesiensis*
GTT1		Toxi1	*G. toxicus*	*G. toxicus*
RIK13		Toxi2	*G. toxicus*	*G. toxicus*
HIT-0	Tahiti, French Polynesia	Toxi3	*G. toxicus*	*G. toxicus*
BP Mar 10-23	St. Thomas, USVI	Silv1	*G. silvae*	*G. silvae*
FC May 10-9	St. Thomas, USVI	Silv2	*G. silvae*	*G. silvae*
SH Apr 11-1	St. Thomas, USVI	Silv 3	*G. silvae*	*G. silvae*

Table 1: (continued)

TRL23	Florida Keys, FL, USA	Silv 4	*G. silvae*	*G. silvae*
KW070922-1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Scab1	*G. scabrosus*	*G. scabrosus*
KW070922-2	Kashiwa-jima Island, Otsuki, Kochi, Japan	Scab2	*G. scabrosus*	*G. scabrosus*
TO80908-1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Scab3	*G. scabrosus*	*G. scabrosus*

Results

Virtual digestion of *Gambierdiscus* DNA sequences predicted that the BsrI restriction enzyme could distinguish the *Gambierdiscus* species in the GCR. *In silico* restriction digest using this enzyme generated a specific number of bands and fragment sizes that should provide differentiation for each *Gambierdiscus* species (Table 2). To test the *in silico* RFLP assay, I first used a collection of "known" DNAs from 11 *Gambierdiscus* species where the identity had been independently determined by DNA sequencing. Amplification of the LSU rRNA D1-D2 region of these *Gambierdiscus* DNAs using the primer pair D1R and D2C yielded a single band of approximately 730 bp, although in a few cases there was also a second band nearly of the same size. In some cases, the number and size of the bands predicted from the *in silico* digestion showed variation with the profiles predicted by the virtual BsrI digestion (Table 2). For example, the *G. belizeanus* virtual digest showed two bands, but six bands were observed in the gel from the PCR-RFLP method. Also, the predicted banding patterns in *G. carolinianus*, *G. caribaeus*, *G. australes*, *G. pacificus* and *G. toxicus* displayed one extra band of small size that was not observed in the gel. On the other hand, *G. silvae*, *G.* ribotype2, *G. carpenteri,* and *G. polynesiensis* the number of predicted bands was the same as the number of observed bands, with similar fragment size.

Digestion of the D1-D2 PCR products with BsrI produced unique fragment banding patterns for all species except for *G. pacificus* and *G. toxicus*. With this exception, the number and size of the fragments from the different species are distinct, making it easy to differentiate between them (Table2). As can be seen in Figure 2, digestion with BsrI produced one band in *G. carolinianus*, two bands in *G. silvae, G.* ribotype2 and *G.*

carpenteri, 3 bands in *G. caribaeus* and 6 bands in *G. belizeanus* with different band sizes in each species.

While there are a few similarities in banding patterns, RFLP patterns permitted the differentiation of the species present in the GCR from Pacific *Gambierdiscus* species (Figure 3). *G. pacificus* and *G. toxicus* showed the same number and size in the pattern of bands. Profiles of *G. carolinianus* and *G. polynesiensis* showed a similar profile with a single band but with a small difference in its size. *G. carolinianus* showed a second blurry band around 600 bp, which can be used to discriminate from *G. polynesiensis*. Within each species the pattern of bands was consistent between multiple strains evaluated (Figure 2, 4, 5, 6, and 7).

Table 2. Predicted and observed fragment sizes (bp) for each of the 11 *Gambierdiscus* species digested with BsrI. D1-D2 region sequences were used to predict the fragment sizes in a virtual BsrI digestion. Bold text represent the species found in the GCR.

Species	Geographic distribution	Abbreviation	Strain from WHOI	Predicted Fragment size (WHOI)	Fragment size(bp) in this study
G. carolinianus	**Atlantic**	**Caro**	**carolNOAA6_1_6**	**650, 41**	**650**
G. belizeanus	**Atlantic**	**Beli**	**beliNOAA5_1_8**	**425, 321**	**515, 318, 216, 196, 183, 162**
G. silvae	**Atlantic**	**Silv**	**ribotype1-2**	**485, 181**	**515, 210**
***G.* ribotype 2**	**Atlantic**	**Ribo2**	**ribotype 2-2**	**404, 304**	**420, 310**
G. caribaeus	**Atlantic/Pacific**	**Cari**	**cariNOAA11_4**	**260, 210, 145, 61**	**260, 220, 160**
G. carpenteri	**Atlantic/Pacific**	**Carp**	**carpNOAA1_5**	**355, 319**	**355, 310**
G. australes	Pacific	Aust	australes clone RAV92_1	331, 219, 98, 52	320, 210, 125
G. pacificus	Pacific	Paci	pacifHO91_4	323, 211, 176, 69	317, 215, 185
G. polynesiensis	Pacific	Poly	polyTB92_3	706 (No Cut Sites)	730
G. scabrosus	Pacific	Scab	No sequence	No sequence	575, 465, 325, 243, 215, 170, 143
G. toxicus	Pacific	Toxi	toxiTUR_4	323, 202, 175, 67	320, 210, 190

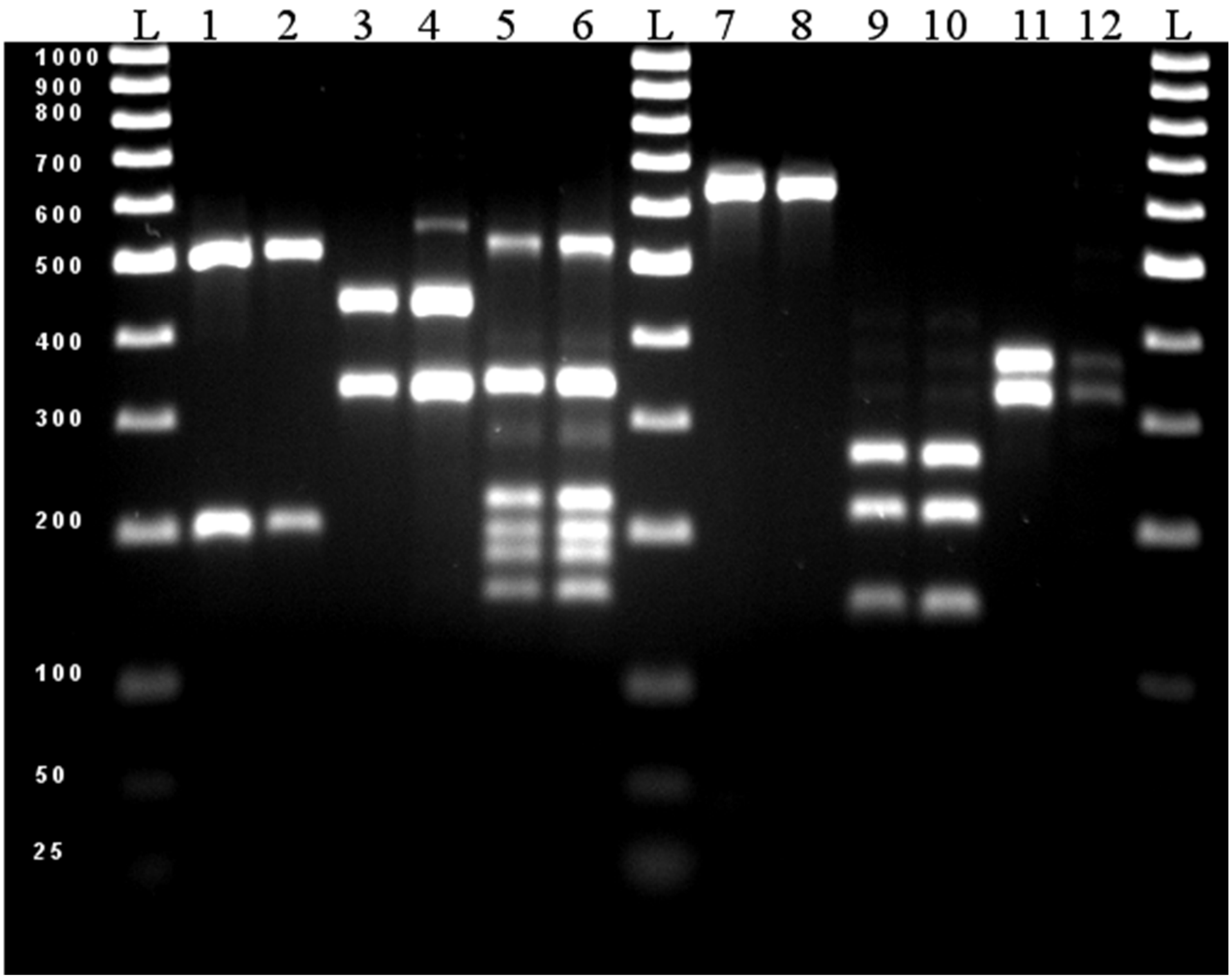

Figure 2. BsrI-digested PCR products of the LSU rRNA D1-D2 region from the six species of *Gambierdiscus* found in the Greater Caribbean Region. Lane 1-2 *G. silvae* (strains silv2 and silv4), lane 3-4 *G.* ribotype2 (strains ribo2-2 and ribo2-4), lane 5-6 *G.belizeanus* (strains beli5 and beli7), lane 7-8 *G. carolinianus* (strains caro1 and caro4), lane 9-10 *G. caribaeus* (strains cari7 and cari8), lane 11-12 *G. carpenteri* (strains carp3 and carp4), and Lane L 100bp PCR DNA Ladder. Strain designations are in Table 1.

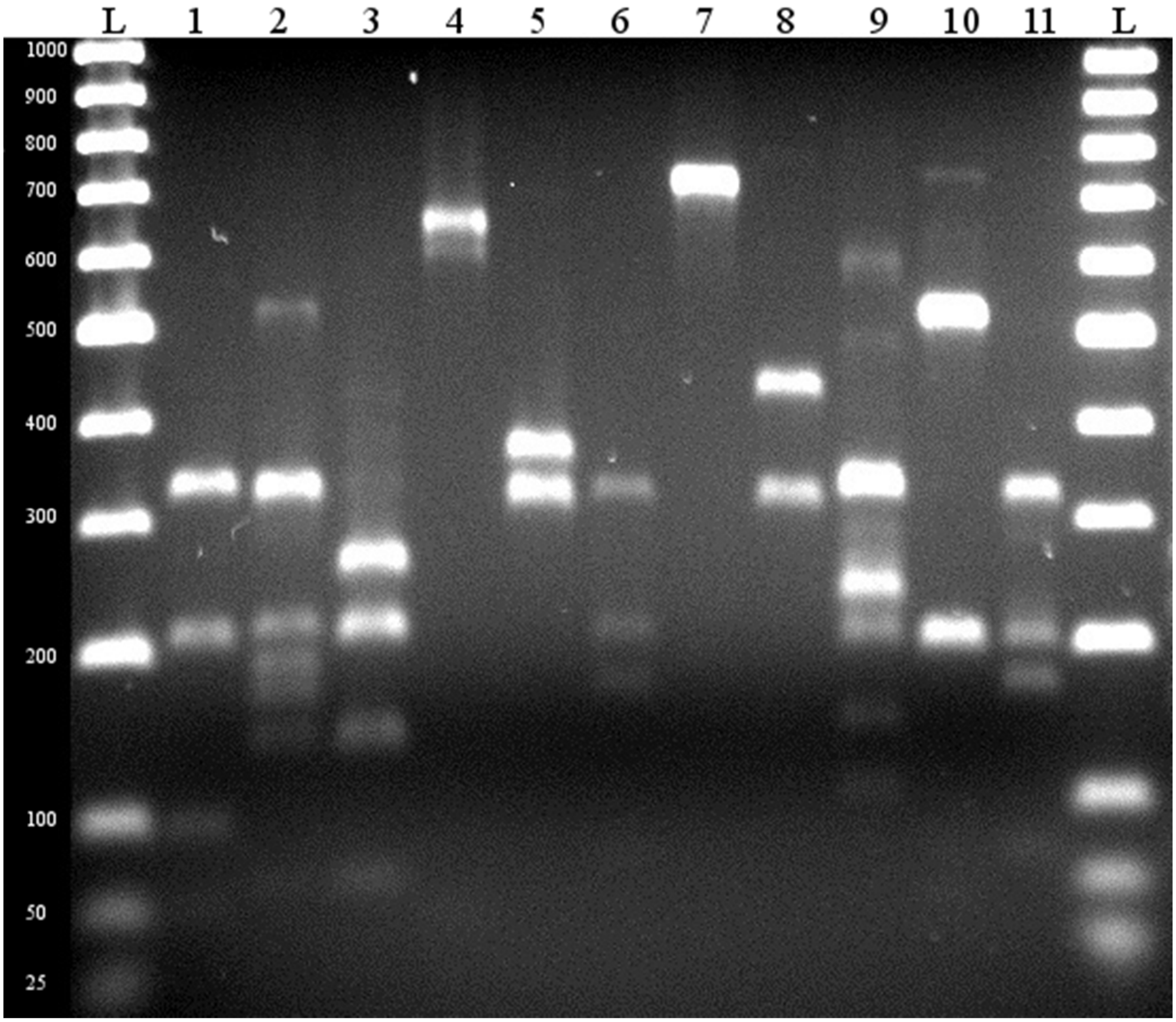

Figure 3. Restriction patterns of BsrI-digested PCR products of the LSU D1-D2 region from eleven *Gambierdiscus* species. 1. *G. australes* (aust2), 2. *G. belizeanus* (beli5), 3. *G. caribaeus* (cari3), 4. *G. carolinianus* (caro2), 5. *G. carpenteri* (carp2), 6. *G. pacificus* (paci1), 7. *G. polynesiensis* (poly3), 8. *G.* ribotype 2 (ribo2-1), 9. *G. scabrosus* (scab1), 10. *G. silvae* (silv4), 11. *G. toxicus* (toxi1), Lane L 100bp PCR DNA Ladder. Strain designations are in Table 1.

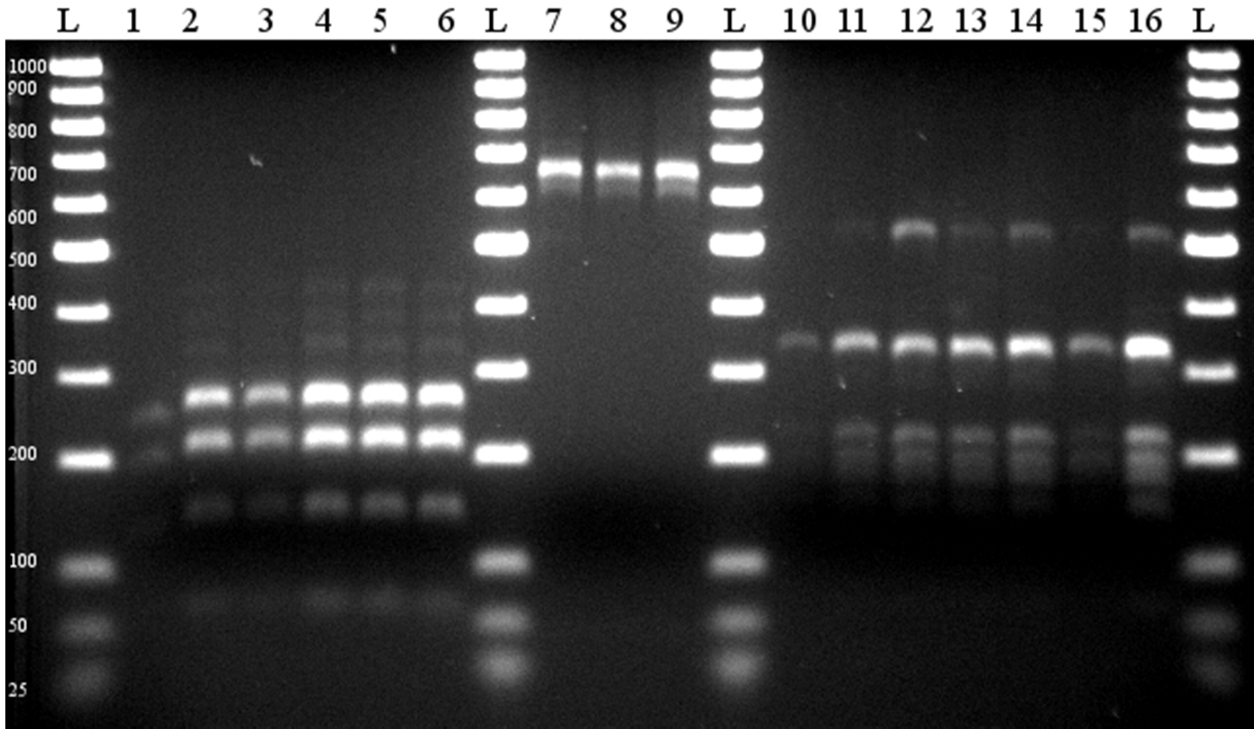

Figure 4. Restriction patterns of BsrI-digested PCR of the LSU D1-D2 region from multiple strains of three *Gambierdiscus* species. Lane 1-6 *G. caribaeus* (strains cari2, cari3, cari5, cari1, cari6 and cari4), Lane 7-8 *G. carolinianus* (strains caro1, caro2 and caro3), Lane 10-16 *G. belizeanus* (strains beli1, beli2, beli3, beli4, beli5, beli6 and beli7); Lane L 100bp PCR DNA Ladder. Strain designations are in Table 1.

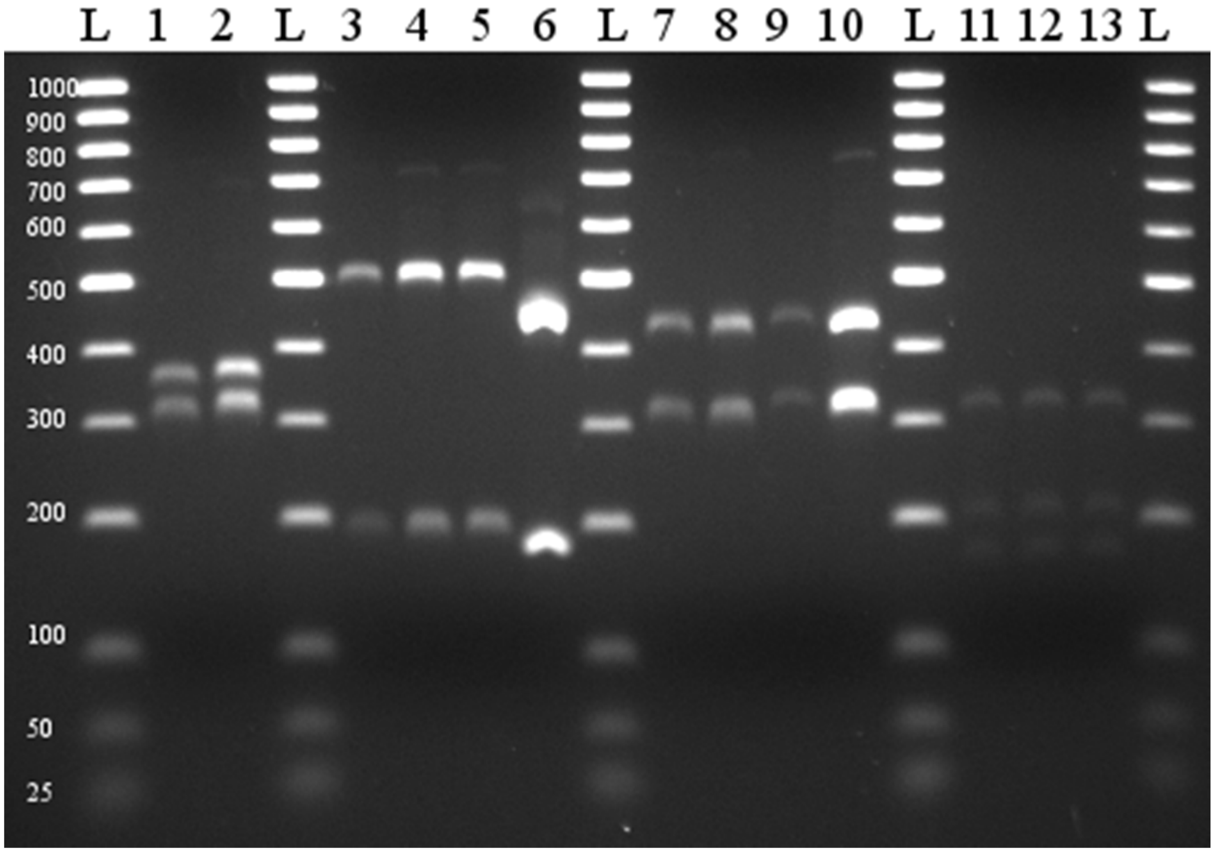

Figure 5. Restriction patterns of BsrI-digested PCR of the LSU D1-D2 region from multiple strains of four *Gambierdiscus* species. Lane 1-2 *G. carpenteri* (strains carp1 and carp2), Lane 3-6 *G. silvae* (strains silv1, silv2, silv3 and silv4), Lane 7-10 *G.* ribotype 2 (strains ribo2-1, ribo2-2, ribo2-3, and ribo2-4), Lane 12-14 *G. pacificus* (strains paci1, paci2, and paci3), Lane L 100bp PCR DNA Ladder. Strain designations are in Table 1.

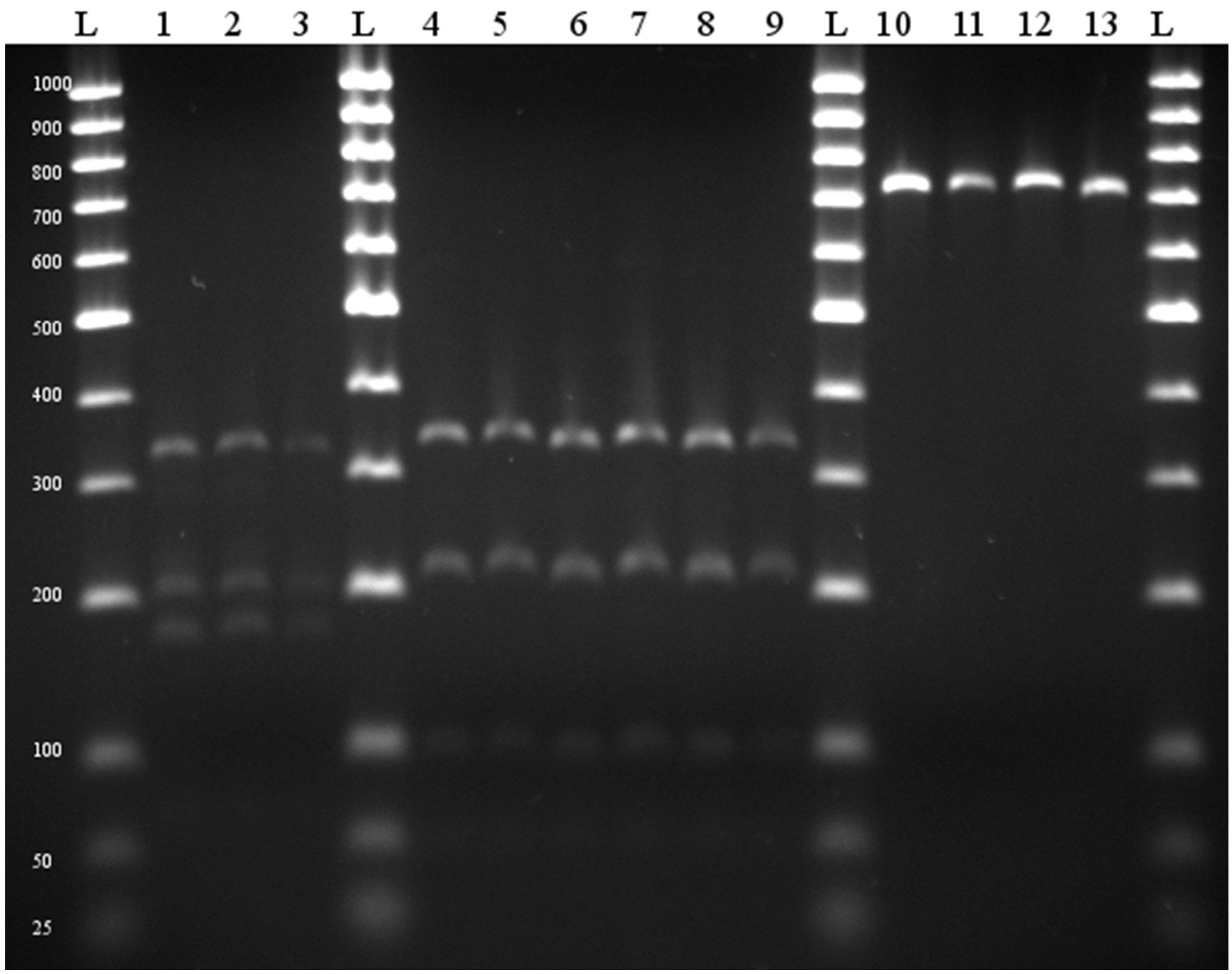

Figure 6. Restriction patterns of BsrI-digested PCR of the LSU D1-D2 region from four *Gambierdiscus* species. Lane 1-3 *G. toxicus* (strains toxi1, toxi2 and toxi3), Lane 4-9 *G. australes* (strains aust1, aust2, aust3, aust4, aust5, and aust6) Lane 10-13 *G. polynesiensis* (strains poly1, poly2, poly3 and poly4), Lane L 100bp PCR DNA Ladder. Strain designations are in Table 1.

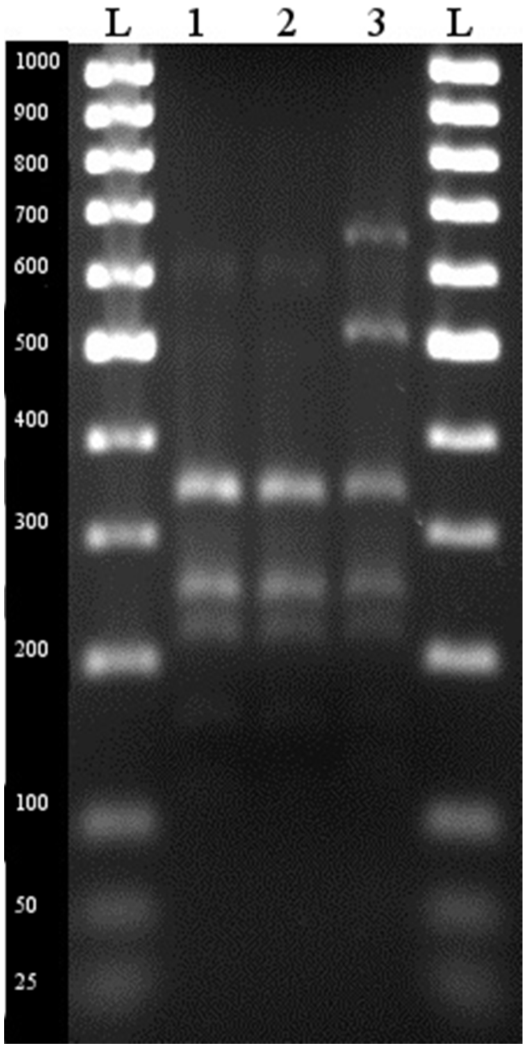

Figure 7. Restriction patterns from BsrI-digested PCR of the LSU D1-D2 region of *G. scabrosus.* Lane 1-3 strains scab1, scab2 and scab3. Lane L 100bp PCR DNA Ladder.

After validation, the utility of the BsrI RFLP method was tested using strains cultured from field collections from the U.S. Virgin Islands. A total of 1154 single cells were isolated, resulting in 496 cultured strains that were analyzed by PCR-RFLP. This corresponded to a 43% culture success rate with this single cell isolation method. From the 96 well cell culture plate, 70% grew sufficiently that they were transferred to small volume (5 ml) culture, and 62% of those were transferred to standard (25 ml) culture. The RFLP patterns of all isolates matched one of the six species that have been reported from the GCR: *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. belizeanus*, *G. silvae* and *G.* ribotype 2. I did not observe any RFLP patterns that matched to those species considered to be restricted to the Pacific: *G. australes*, *G. pacificus*, *G. polynesiensis*, *G. scabrosus*, and *G. toxicus*. All the isolates exhibited a banding pattern that could be matched to a type pattern, i.e. no novel banding patterns were observed. In my culture collection, *G. carolinianus* and *G. caribaeus* were the most common species (40% and 33% of strains, respectively, Figure 8). Only 2 strains of *G. ribotype2* (0.4%) were found. Other species represented a low percentage of strains identified: *G. belizeanus* (10.1%), *G. carpenteri* (5.2%), and *G. silvae* (5.6%). A few individuals (6%) were not identified because they did not produce successful PCR products.

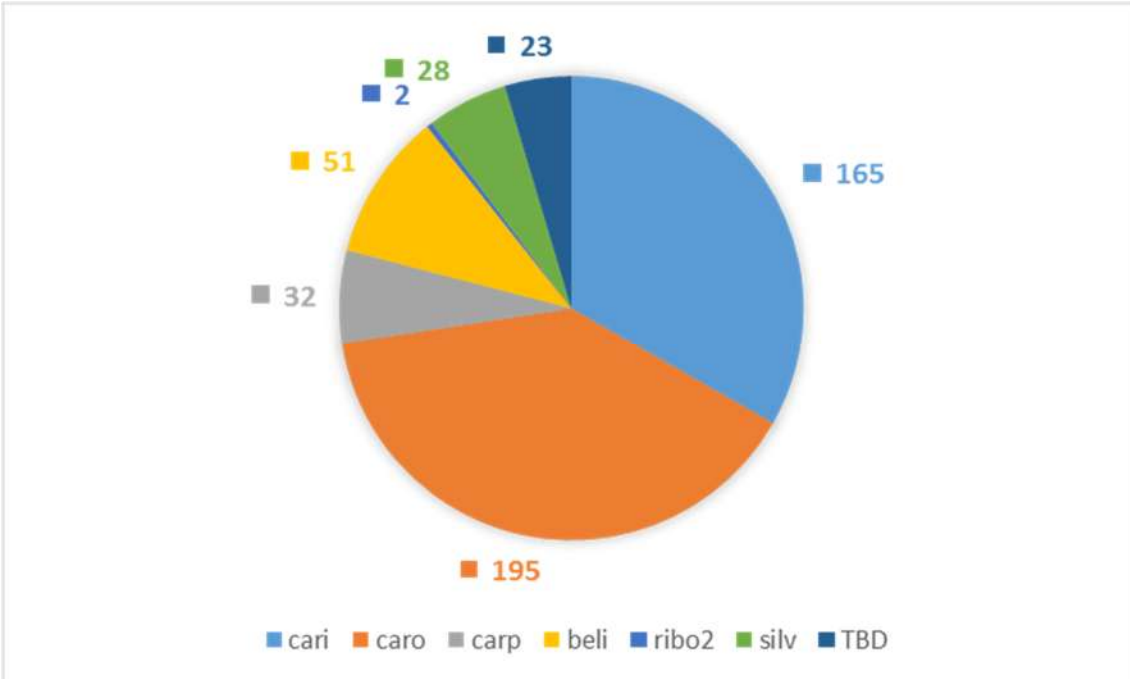

Figure 8. Number of strains of each species from the U.S.-Virgin Islands identified with the PCR-RFLP method. cari: *G. caribaeus*, caro: *G. carolinianus*, carp: *G. carpenteri*, beli: *G. belizeanus*, ribo2: *G*. ribotype 2, silv: *G. silvae* and TBD: to be determined.

Discussion

This study developed and validated a polymerase chain reaction - restriction fragment length polymorphism assay using the LSU rRNA D1-D2 hypervariable regions to distinguish among the species of the dinoflagellate *Gambierdiscus* found in the GCR. This PCR-RFLP method using the BsrI restriction enzyme enables a reliable identification of the six species of *Gambierdiscus* presented in the region. PCR-RFLP profiles using LSU rRNA found in this study for each of the six species corresponded with their morphological cell description, as they have been previously reported (Litaker *et al*. 2009). Also, the PCR-RFLP profile supports the geographic distribution of this genus (Litaker *et al*. 2010), as the GCR *Gambierdiscus* species showed distinct differences to PCR-RFLP profiles from the Pacific *Gambierdiscus* species evaluated, and the 473 USVI isolates typed by RFLP were all identified as one of the six species known from the Caribbean.

The LSU rRNA D1-D2 region is a valuable molecular marker due to its sequence variation allow to discriminate the six species of *Gambierdiscus* in the GCR. The rRNA genes occur in high copy numbers in dinoflagellates (Galluzzi *et al*. 2010), and the D1-D2 region from all the *Gambierdiscus* samples were stably amplified. After digestion, some *Gambierdiscus* spp. profiles showed extra bands among strains of the same species (Figure 2, 5, 7), possibly caused by pseudogene sequences (Litaker *et al*. 2009), PCR mispriming, or degradation products. However, this should not interfere with their identification. For example, the variation seen in *G. silvae* with the strain silv4 would not prevent routine identification of this species because it retains its characteristic banding pattern – 2 bands, widely spaced – even though the fragment sizes are slightly smaller than in the other strains. Species-specific restriction patterns produced by BsrI restriction enzyme digestion were enough to clearly separate species from each other. LSU rRNA D1-D2 region sequences and RFLP have been used successfully in others groups of dinoflagellates, as *Alexandrium*, *Dinophysis*, and *Karenia*, to assess the genetic diversity and the intra- and interspecific relationships (Scholin *et al*. 1994, Guillou *et al*. 2002, Hart *et al*. 2007)). This study shows that *Gambierdiscus* is another group where the LSU rRNA D1-D2 region can provide interspecific resolution.

This method can be reliably used for field identification of *Gambierdiscus* species in the GCR, as the six Caribbean species are easily differentiated from the known Pacific species. Similarity in banding patterns within Pacific species and between Pacific and Caribbean species limits its use for discriminating all species of *Gambierdiscus*. The BsrI enzyme produces identical RFLP patterns for the *G. toxicus* and *G. pacificus*. Both of these species are known from the Pacific only, thus their separation would require the use of another method. Phylogenetic analysis of LSU rRNA gene shows that this species pair exhibits a low level of genetic differentiation, which is here reflected in a lack of restriction enzyme polymorphism (Chinain *et al*. 1999, Litaker *et al*. 2009, and Fraga and Rodríguez 2014).

These same studies detected little genetic variation between *G. carolinianus* and *G. polynesiensis*, and these two species showed similar BsrI banding patterns - both species showed a single band with a similar size. These two species are thought to be restricted to different ocean regions (Caribbean and Pacific respectively), thus their similarities should not present a major difficulty for studies conducted within the GCR. However, given that the geographic distributions are known only from cultured isolates, it is important that any method be able to detect instances of 'non-Caribbean' species that may be first reports from the field or invasions/introductions. To differentiate *G. polynesiensis* and *G. carolinianus*, it is necessary to precisely size the RFLP fragments or compare them side-by-side on a gel. *G. polynesiensis* showed one band with the same size as the intact D1-D2 amplicon because there are no BsrI sites within its D1-D2 sequence. On the other hand, the D1-D2 region from *G. carolinianus* has one BsrI cut site, which produces a bright band of 650 bp and a smaller fragment (predicted 41bp) that was generally not visible on the gel due to its size. Thus, *G. polynesiensis* shows one band between 700-800 bp and *G. carolinianus* shows one band between 600-700 pb.

In the present study, the PCR-RFLP method was successfully applied to identify unknown *Gambierdiscus* species from field samples taken in the US Virgin islands. This is an important advance in the study of the *Gambierdiscus* in the GCR. This methodology allows identification of species using molecular information from recent studies from this genus. The proportion of *Gambierdiscus* species amongst my cultured isolates does not represent the abundance of cells in the field, but instead denotes the efficiency of single cell isolation and culturing for various species. This method provides an easy and efficient way to identify isolates cultured for specific purposes, e.g. physiological or population genetic studies. However, it is important to be able to determine the abundance and distribution of *Gambierdiscus* cells and species in the field, which is not possible using this single cell isolation technique. This necessitates the development of other methods that can be used directly without cell cultivation. The culture methods used here work well for *G. caribaeus* and *G. carolinianus*, but it is difficult to say whether other species were less abundant because of culture bias or lower population numbers in the field. In future works, it may be useful to test other culture media to improve the isolation of the species with low representation with this method.

The new PCR-RFLP method developed in this study is a practical, useful, quick, cheap and reliable assay to identity *Gambierdiscus* species in the GCR; and it works in conjunction with basic morphological identification of *Gambierdiscus* to characterize the diversity of species in this genus. This method could be used in laboratories where identification of *Gambierdiscus* species is a routine task. This method also expands the tools available to researchers and managers engaged in monitoring activities and ecological studies of toxic dinoflagellates.

Appendices

APPENDIX A - LIST OF GAMBIERDISCUS SPECIES AND RIBOTYPES WITH THEIR REFERENCES

Table A1. List of the known Gambierdiscus species and ribotypes with their references, isolation location, and synonymies.

Genus	Species	Reference	Locality	Synonymy
Gambierdiscus	*G. australes*	Chinain *et al*. 1999	Rurua, Raivavae Island	
	G. balechii	Fraga *et al*. 2016	Manado, Indonesia	
	G. belizeanus	Faust 1995	Belize, Caribbean Sea	
	G. caribaeus	Litaker *et al*. 2009	Carrie Bow Cay, Belize	
	G. carolinianus	Litaker *et al*. 2009	Cape Fear, North Carolina, USA	
	G. carpenteri	Litaker *et al*. 2009	South Water Cay, Belize	
	G. excentricus	Fraga *et al*. 2011	Canary Islands, Spain	
	G. pacificus	Chinain *et al*. 1999	Otepa, Hao Island	
	G. polynesiensis	Chinain *et al*. 1999	Mataura, Tubuai island	
	G. ribotype 2	Litaker *et al*. 2010	Martinique, Caribbean Sea	
	G. scabrosus	Nishimura *et al*. 2014	Kashiwa-jima Island, Japan	*G*. sp. type 1
	G. silvae	Fraga and Rodríguez 2014	Caribbean Sea	*G*. sp. ribotype 1
	G. toxicus	Adachi and Fukuyo 1979	Gambier Island, French Polynesia	
	G. sp. type 2	Kuno *et al*. 2010	Japan	
	G. sp. type 3	Nishimura *et al*. 2013	Japan	
	G. sp. type 4	Xu *et al*. 2014	Marakei, Republic of Kiribati	
	G. sp. type 5	Xu *et al*. 2014	Marakei, Republic of Kiribati	
	G. sp. type 6	Xu *et al*. 2014	Marakei, Republic of Kiribati	
Fukuyoa	*F. yasumotoi*	Holmes 1998	Pulau Hantu - Singapore	*G. yasumotoi*
	F. ruetzleri	Litaker *et al*. 2009	South Water Cay, Belize	*G. ruetzleri*

APPENDIX B - STRAINS OF GAMBIERDISCUS SPECIES ISOLATED

Table B1. Strains of Gambierdiscus species isolated from ST. Thomas –US Virgin Islands and tested in this study. TBD: to be determined

Species	Strain name	Locality	Isolation date
G. belizeanus	1310FC-1	Flat Cay	10/22/13
G. belizeanus	1310FC-3	Flat Cay	10/22/13
G. belizeanus	1310FC-5	Flat Cay	10/22/13
G. belizeanus	1310FC-8	Flat Cay	10/22/13
G. belizeanus	1310FC-9	Flat Cay	10/22/13
G. belizeanus	1311SH-3	Seahorse	11/25/13
G. belizeanus	1311SH-6	Seahorse	11/25/13
G. belizeanus	1311SH-7	Seahorse	11/25/13
G. belizeanus	1311SH-8	Seahorse	11/25/13
G. belizeanus	1311SH-9	Seahorse	11/25/13
G. belizeanus	1311SH-10	Seahorse	11/25/13
G. belizeanus	1311SH-11	Seahorse	11/25/13
G. belizeanus	1402FC-3	Flat Cay	2/12/14
G. belizeanus	1402FC-7	Flat Cay	2/12/14
G. belizeanus	1403BB-9	Benner Bay	3/13/14
G. belizeanus	1404FC-10	Flat Cay	4/15/14
G. belizeanus	1404FC-11	Flat Cay	4/15/14
G. belizeanus	1404SH-1	Seahorse	4/15/14
G. belizeanus	1404BP2-2	Black Point	4/15/14
G. belizeanus	1404BP2-3	Black Point	4/15/14
G. belizeanus	1404BP2-5	Black Point	4/15/14
G. belizeanus	1404BP2-10	Black Point	4/15/14
G. belizeanus	1404BP2-12	Black Point	4/15/14
G. belizeanus	1404BB2-6	Benner Bay	4/15/14
G. belizeanus	1404BB2-8	Benner Bay	4/15/14
G. belizeanus	1404BB2-11	Benner Bay	4/15/14
G. belizeanus	1407SH-4	Seahorse	7/8/14
G. belizeanus	1407BP-14	Black Point	7/8/14
G. belizeanus	1409FC2-8	Flat Cay	9/2/14
G. belizeanus	1409BP2-4	Black Point	9/2/14
G. belizeanus	1409BB2-2	Benner Bay	9/2/14
G. belizeanus	1409BB2-6	Benner Bay	9/2/14
G. belizeanus	1409BB2-7	Benner Bay	9/2/14

Table B1: (continued)

G. belizeanus	1409BB2-9	Benner Bay	9/2/14
G. belizeanus	1409BB2-10	Benner Bay	9/2/14
G. belizeanus	1501SH-10	Seahorse	1/14/15
G. belizeanus	1504BB-9	Benner Bay	4/13/15
G. belizeanus	1504FC-1	Flat Cay	4/13/15
G. belizeanus	1507BB-12	Benner Bay	7/7/15
G. belizeanus	1507BB-18	Benner Bay	7/7/15
G. belizeanus	1507BB-19	Benner Bay	7/7/15
G. belizeanus	1507BB-21	Benner Bay	7/7/15
G. belizeanus	1507FC-2	Flat Cay	7/7/15
G. belizeanus	1507FC-4	Flat Cay	7/7/15
G. belizeanus	1507FC-18	Flat Cay	7/7/15
G. belizeanus	1507FC-19	Flat Cay	7/7/15
G. belizeanus	1507FC-22	Flat Cay	7/7/15
G. belizeanus	1507FC-31	Flat Cay	7/7/15
G. belizeanus	1507FC-32	Flat Cay	7/7/15
G. belizeanus	1507FC-34	Flat Cay	7/7/15
G. belizeanus	1507FC-38	Flat Cay	7/7/15
G. caribaeus	1308SH2-1	Seahorse	8/26/13
G. caribaeus	1308BB1-10	Benner Bay	8/26/13
G. caribaeus	1308SH2-3	Seahorse	8/26/13
G. caribaeus	1308SH2-5	Seahorse	8/26/13
G. caribaeus	1308BB1-4	Benner Bay	8/26/13
G. caribaeus	1308BB1-7	Benner Bay	8/26/13
G. caribaeus	1310FC-7	Flat Cay	10/22/13
G. caribaeus	1310SH-1	Seahorse	10/22/13
G. caribaeus	1310SH-2	Seahorse	10/22/13
G. caribaeus	1310BB-2	Benner Bay	10/22/13
G. caribaeus	1310BB-3	Benner Bay	10/22/13
G. caribaeus	1310BB-5	Benner Bay	10/22/13
G. caribaeus	1310BP-2	Black Point	10/22/13
G. caribaeus	1310BP-3	Black Point	10/22/13
G. caribaeus	1310BP-4	Black Point	10/22/13
G. caribaeus	1310BP-5	Black Point	10/22/13
G. caribaeus	1310BP-6	Black Point	10/22/13
G. caribaeus	1310BP-7	Black Point	10/22/13
G. caribaeus	1310BP-8	Black Point	10/22/13

Table B1: (continued)

G. caribaeus	1311FC-2	Flat Cay	11/25/13
G. caribaeus	1401SH-2	Seahorse	1/16/14
G. caribaeus	1401SH-3	Seahorse	1/16/14
G. caribaeus	1401SH-4	Seahorse	1/16/14
G. caribaeus	1401SH-5	Seahorse	1/16/14
G. caribaeus	1401SH-6	Seahorse	1/16/14
G. caribaeus	1401SH-7	Seahorse	1/16/14
G. caribaeus	1401SH-9	Seahorse	1/16/14
G. caribaeus	1401SH-10	Seahorse	1/16/14
G. caribaeus	1401SH-11	Seahorse	1/16/14
G. caribaeus	1401BB-8	Benner Bay	1/16/14
G. caribaeus	1401BP-2	Black Point	1/16/14
G. caribaeus	1401BP-3	Black Point	1/16/14
G. caribaeus	1401BP-4	Black Point	1/16/14
G. caribaeus	1401BP-6	Black Point	1/16/14
G. caribaeus	1401BP-7	Black Point	1/16/14
G. caribaeus	1401BP-8	Black Point	1/16/14
G. caribaeus	1401BP-13	Black Point	1/16/14
G. caribaeus	1402FC-4	Flat Cay	2/12/14
G. caribaeus	1402BP-4	Black Point	2/12/14
G. caribaeus	1402BP-6	Black Point	2/12/14
G. caribaeus	1402BP-11	Black Point	2/12/14
G. caribaeus	1402SH-2	Seahorse	2/12/14
G. caribaeus	1402SH-3	Seahorse	2/12/14
G. caribaeus	1402SH-5	Seahorse	2/12/14
G. caribaeus	1402SH-6	Seahorse	2/12/14
G. caribaeus	1402SH-7	Seahorse	2/12/14
G. caribaeus	1402SH-9	Seahorse	2/12/14
G. caribaeus	1402SH-10	Seahorse	2/12/14
G. caribaeus	1402SH-13	Seahorse	2/12/14
G. caribaeus	1403FC-7	Flat Cay	3/13/14
G. caribaeus	1403BP-9	Black Point	3/13/14
G. caribaeus	1403SH-7	Seahorse	3/13/14
G. caribaeus	1404SH-2	Seahorse	4/15/14
G. caribaeus	1404BP2-13	Black Point	4/15/14
G. caribaeus	1404BP2-15	Black Point	4/15/14
G. caribaeus	1405SH-3	Seahorse	5/14/14

Table B1: (continued)

G. caribaeus	1406FC-2	Flat Cay	6/10/14
G. caribaeus	1406BB-2	Benner Bay	6/10/14
G. caribaeus	1407SH-1	Seahorse	7/8/14
G. caribaeus	1407SH-2	Seahorse	7/8/14
G. caribaeus	1407SH-9	Seahorse	7/8/14
G. caribaeus	1407BB-2	Benner Bay	7/8/14
G. caribaeus	1407BB-5	Benner Bay	7/8/14
G. caribaeus	1407FC-9	Flat Cay	7/8/14
G. caribaeus	1407FC-11	Flat Cay	7/8/14
G. caribaeus	1407BP-8	Black Point	7/8/14
G. caribaeus	1407BP-10	Black Point	7/8/14
G. caribaeus	1408BP-2	Black Point	8/6/14
G. caribaeus	1408BP-5	Black Point	8/6/14
G. caribaeus	1408BP-6	Black Point	8/6/14
G. caribaeus	1408SH-1	Seahorse	8/6/14
G. caribaeus	1408SH-3	Seahorse	8/6/14
G. caribaeus	1408SH-6	Seahorse	8/6/14
G. caribaeus	1408SH-7	Seahorse	8/6/14
G. caribaeus	1408BB-1	Benner Bay	8/6/14
G. caribaeus	1409BP-4	Black Point	9/2/14
G. caribaeus	1409SH-1	Seahorse	9/2/14
G. caribaeus	1409BB-2	Benner Bay	9/2/14
G. caribaeus	1409BB-4	Benner Bay	9/2/14
G. caribaeus	1409FC2-3	Flat Cay	9/2/14
G. caribaeus	1409BP2-3	Black Point	9/2/14
G. caribaeus	1409SH2-1	Seahorse	9/2/14
G. caribaeus	1409SH2-2	Seahorse	9/2/14
G. caribaeus	1409SH2-3	Seahorse	9/2/14
G. caribaeus	1409SH2-4	Seahorse	9/2/14
G. caribaeus	1409SH2-6	Seahorse	9/2/14
G. caribaeus	1409SH2-7	Seahorse	9/2/14
G. caribaeus	1409SH2-8	Seahorse	9/2/14
G. caribaeus	1409SH2-9	Seahorse	9/2/14
G. caribaeus	1409SH2-10	Seahorse	9/2/14
G. caribaeus	1409BB2-3	Benner Bay	9/2/14
G. caribaeus	1410FC-2	Flat Cay	10/22/14
G. caribaeus	1410FC-4	Flat Cay	10/22/14

Table B1: (continued)

G. caribaeus	1410FC-5	Flat Cay	10/22/14
G. caribaeus	1410FC-10	Flat Cay	10/22/14
G. caribaeus	1410SH-1	Seahorse	10/22/14
G. caribaeus	1410SH-4	Seahorse	10/22/14
G. caribaeus	1410SH-5	Seahorse	10/22/14
G. caribaeus	1410SH-7	Seahorse	10/22/14
G. caribaeus	1410SH-8	Seahorse	10/22/14
G. caribaeus	1410SH-9	Seahorse	10/22/14
G. caribaeus	1410SH-10	Seahorse	10/22/14
G. caribaeus	1410SH-11	Seahorse	10/22/14
G. caribaeus	1410SH-12	Seahorse	10/22/14
G. caribaeus	1410SH-13	Seahorse	10/22/14
G. caribaeus	1410SH-14	Seahorse	10/22/14
G. caribaeus	1410SH-15	Seahorse	10/22/14
G. caribaeus	1410SH-16	Seahorse	10/22/14
G. caribaeus	1410BP-4	Black Point	10/22/14
G. caribaeus	1410BP-7	Black Point	10/22/14
G. caribaeus	1410BP-8	Black Point	10/22/14
G. caribaeus	1410BP-11	Black Point	10/22/14
G. caribaeus	1410BP-12	Black Point	10/22/14
G. caribaeus	1410BP-14	Black Point	10/22/14
G. caribaeus	1410BP-16	Black Point	10/22/14
G. caribaeus	1410BP-17	Black Point	10/22/14
G. caribaeus	1410BP-18	Black Point	10/22/14
G. caribaeus	1410BP-19	Black Point	10/22/14
G. caribaeus	1501SH-2	Seahorse	1/14/15
G. caribaeus	1501SH-3	Seahorse	1/14/15
G. caribaeus	1501SH-4	Seahorse	1/14/15
G. caribaeus	1501SH-5	Seahorse	1/14/15
G. caribaeus	1501SH-6	Seahorse	1/14/15
G. caribaeus	1501SH-7	Seahorse	1/14/15
G. caribaeus	1501SH-8	Seahorse	1/14/15
G. caribaeus	1501SH-11	Seahorse	1/14/15
G. caribaeus	1501BP-2	Black Point	1/14/15
G. caribaeus	1501BP-4	Black Point	1/14/15
G. caribaeus	1502FC-3	Flat Cay	2/10/15
G. caribaeus	1502BP-1	Black Point	2/10/15

Table B1: (continued)

G. caribaeus	1503BP-5	Black Point	3/17/15
G. caribaeus	1503BP-7	Black Point	3/17/15
G. caribaeus	1503FC-5	Flat Cay	3/17/15
G. caribaeus	1503BB-7	Benner Bay	3/17/15
G. caribaeus	1503BB-9	Benner Bay	3/17/15
G. caribaeus	1503BB-10	Benner Bay	3/17/15
G. caribaeus	1503SH-4	Seahorse	3/17/15
G. caribaeus	1503SH-5	Seahorse	3/17/15
G. caribaeus	1503SH-6	Seahorse	3/17/15
G. caribaeus	1504BB-5	Benner Bay	4/13/15
G. caribaeus	1504BB-14	Benner Bay	4/13/15
G. caribaeus	1504BP-2	Black Point	4/13/15
G. caribaeus	1504BP-3	Black Point	4/13/15
G. caribaeus	1504BP-6	Black Point	4/13/15
G. caribaeus	1504BP-7	Black Point	4/13/15
G. caribaeus	1504BP-9	Black Point	4/13/15
G. caribaeus	1504BP-13	Black Point	4/13/15
G. caribaeus	1504FC-2	Flat Cay	4/13/15
G. caribaeus	1504FC-4	Flat Cay	4/13/15
G. caribaeus	1504FC-5	Flat Cay	4/13/15
G. caribaeus	1504FC-7	Flat Cay	4/13/15
G. caribaeus	1504SH-2	Seahorse	4/13/15
G. caribaeus	1504SH-4	Seahorse	4/13/15
G. caribaeus	1504SH-11	Seahorse	4/13/15
G. caribaeus	1505BP-2	Black Point	5/11/15
G. caribaeus	1505BP-9	Black Point	5/11/15
G. caribaeus	1505BB-3	Benner Bay	5/11/15
G. caribaeus	1505BB-4	Benner Bay	5/11/15
G. caribaeus	1505BB-12	Benner Bay	5/11/15
G. caribaeus	1505BB-14	Benner Bay	5/11/15
G. caribaeus	1505SH-2	Seahorse	5/11/15
G. caribaeus	1505SH-3	Seahorse	5/11/15
G. caribaeus	1505SH-10	Seahorse	5/11/15
G. caribaeus	1507BB-20	Benner Bay	7/7/15
G. carolinianus	1308SH2-9	Seahorse	8/26/13
G. carolinianus	1310BP-1	Black Point	10/22/13
G. carolinianus	1401SH-14	Seahorse	1/16/14

Table B1: (continued)

G. carolinianus	1401SH-16	Seahorse	1/16/14
G. carolinianus	1401BB-1	Benner Bay	1/16/14
G. carolinianus	1401BB-2	Benner Bay	1/16/14
G. carolinianus	1402FC-5	Flat Cay	2/12/14
G. carolinianus	1402FC-9	Flat Cay	2/12/14
G. carolinianus	1402BP-1	Black Point	2/12/14
G. carolinianus	1402BP-9	Black Point	2/12/14
G. carolinianus	1402SH-12	Seahorse	2/12/14
G. carolinianus	1402BB-1	Benner Bay	2/12/14
G. carolinianus	1402BB-5	Benner Bay	2/12/14
G. carolinianus	1402BB-14	Benner Bay	2/12/14
G. carolinianus	1403FC-1	Flat Cay	3/13/14
G. carolinianus	1403FC-5	Flat Cay	3/13/14
G. carolinianus	1403FC-6	Flat Cay	3/13/14
G. carolinianus	1403FC-10	Flat Cay	3/13/14
G. carolinianus	1403FC-11	Flat Cay	3/13/14
G. carolinianus	1403FC-12	Flat Cay	3/13/14
G. carolinianus	1403BP-3	Black Point	3/13/14
G. carolinianus	1403BP-4	Black Point	3/13/14
G. carolinianus	1403BP-5	Black Point	3/13/14
G. carolinianus	1403BP-10	Black Point	3/13/14
G. carolinianus	1403SH-4	Seahorse	3/13/14
G. carolinianus	1403SH-6	Seahorse	3/13/14
G. carolinianus	1403BB-3	Benner Bay	3/13/14
G. carolinianus	1403BB-4	Benner Bay	3/13/14
G. carolinianus	1404FC-1	Flat Cay	4/15/14
G. carolinianus	1404FC-3	Flat Cay	4/15/14
G. carolinianus	1404SH-3	Seahorse	4/15/14
G. carolinianus	1404SH-9	Seahorse	4/15/14
G. carolinianus	1404BP2-1	Black Point	4/15/14
G. carolinianus	1404BP2-4	Black Point	4/15/14
G. carolinianus	1404BP2-6	Black Point	4/15/14
G. carolinianus	1404BP2-9	Black Point	4/15/14
G. carolinianus	1404BB2-1	Benner Bay	4/15/14
G. carolinianus	1404BB2-5	Benner Bay	4/15/14
G. carolinianus	1405BP-5	Black Point	5/14/14
G. carolinianus	1405BP-6	Black Point	5/14/14

Table B1: (continued)

G. carolinianus	1405BP-8	Black Point	5/14/14
G. carolinianus	1405BP-9	Black Point	5/14/14
G. carolinianus	1405FC-2	Flat Cay	5/14/14
G. carolinianus	1405FC-4	Flat Cay	5/14/14
G. carolinianus	1405FC-8	Flat Cay	5/14/14
G. carolinianus	1405SH-2	Seahorse	5/14/14
G. carolinianus	1405SH-5	Seahorse	5/14/14
G. carolinianus	1405SH-7	Seahorse	5/14/14
G. carolinianus	1405SH-8	Seahorse	5/14/14
G. carolinianus	1405SH-9	Seahorse	5/14/14
G. carolinianus	1405SH-11	Seahorse	5/14/14
G. carolinianus	1406FC-3	Flat Cay	6/10/14
G. carolinianus	1406FC-7	Flat Cay	6/10/14
G. carolinianus	1406FC-8	Flat Cay	6/10/14
G. carolinianus	1406FC-9	Flat Cay	6/10/14
G. carolinianus	1406FC-11	Flat Cay	6/10/14
G. carolinianus	1406FC-12	Flat Cay	6/10/14
G. carolinianus	1406BP-1	Black Point	6/10/14
G. carolinianus	1406BP-2	Black Point	6/10/14
G. carolinianus	1406BP-3	Black Point	6/10/14
G. carolinianus	1406BP-4	Black Point	6/10/14
G. carolinianus	1406BP-7	Black Point	6/10/14
G. carolinianus	1406BP-10	Black Point	6/10/14
G. carolinianus	1406BP-14	Black Point	6/10/14
G. carolinianus	1406SH-3	Seahorse	6/10/14
G. carolinianus	1406SH-5	Seahorse	6/10/14
G. carolinianus	1406SH-9	Seahorse	6/10/14
G. carolinianus	1406SH-10	Seahorse	6/10/14
G. carolinianus	1406BB-1	Benner Bay	6/10/14
G. carolinianus	1406BB-4	Benner Bay	6/10/14
G. carolinianus	1406BB-6	Benner Bay	6/10/14
G. carolinianus	1406BB-11	Benner Bay	6/10/14
G. carolinianus	1406BB-13	Benner Bay	6/10/14
G. carolinianus	1407SH-3	Seahorse	7/8/14
G. carolinianus	1407BB-7	Benner Bay	7/8/14
G. carolinianus	1407BB-10	Benner Bay	7/8/14
G. carolinianus	1407BB-12	Benner Bay	7/8/14

Table B1: (continued)

G. carolinianus	1407FC-4	Flat Cay	7/8/14
G. carolinianus	1408FC-2	Flat Cay	8/6/14
G. carolinianus	1408FC-3	Flat Cay	8/6/14
G. carolinianus	1408FC-6	Flat Cay	8/6/14
G. carolinianus	1408FC-8	Flat Cay	8/6/14
G. carolinianus	1408FC-9	Flat Cay	8/6/14
G. carolinianus	1408FC-10	Flat Cay	8/6/14
G. carolinianus	1408FC-12	Flat Cay	8/6/14
G. carolinianus	1408FC-13	Flat Cay	8/6/14
G. carolinianus	1408BP-8	Black Point	8/6/14
G. carolinianus	1408BP-12	Black Point	8/6/14
G. carolinianus	1408SH-8	Seahorse	8/6/14
G. carolinianus	1408SH-11	Seahorse	8/6/14
G. carolinianus	1408SH-14	Seahorse	8/6/14
G. carolinianus	1408BB-3	Benner Bay	8/6/14
G. carolinianus	1408BB-4	Benner Bay	8/6/14
G. carolinianus	1409SH2-11	Seahorse	9/2/14
G. carolinianus	1409BB2-4	Benner Bay	9/2/14
G. carolinianus	1410FC-6	Flat Cay	10/22/14
G. carolinianus	1410FC-8	Flat Cay	10/22/14
G. carolinianus	1410SH-17	Seahorse	10/22/14
G. carolinianus	1501BB-1	Benner Bay	1/14/15
G. carolinianus	1501BB-2	Benner Bay	1/14/15
G. carolinianus	1501BB-4	Benner Bay	1/14/15
G. carolinianus	1501BB-5	Benner Bay	1/14/15
G. carolinianus	1501BB-6	Benner Bay	1/14/15
G. carolinianus	1501BP-1	Black Point	1/14/15
G. carolinianus	1501BP-3	Black Point	1/14/15
G. carolinianus	1501BP-5	Black Point	1/14/15
G. carolinianus	1502FC-1	Flat Cay	2/10/15
G. carolinianus	1502FC-2	Flat Cay	2/10/15
G. carolinianus	1502BP-2	Black Point	2/10/15
G. carolinianus	1503BP-1	Black Point	3/17/15
G. carolinianus	1503BP-3	Black Point	3/17/15
G. carolinianus	1503BP-10	Black Point	3/17/15
G. carolinianus	1503BP-11	Black Point	3/17/15
G. carolinianus	1503FC-2	Flat Cay	3/17/15

Table B1: (continued)

G. carolinianus	1503FC-6	Flat Cay	3/17/15
G. carolinianus	1503FC-7	Flat Cay	3/17/15
G. carolinianus	1503FC-8	Flat Cay	3/17/15
G. carolinianus	1503BB-1	Benner Bay	3/17/15
G. carolinianus	1503BB-2	Benner Bay	3/17/15
G. carolinianus	1503BB-3	Benner Bay	3/17/15
G. carolinianus	1503BB-4	Benner Bay	3/17/15
G. carolinianus	1503BB-5	Benner Bay	3/17/15
G. carolinianus	1503BB-11	Benner Bay	3/17/15
G. carolinianus	1503BB-12	Benner Bay	3/17/15
G. carolinianus	1503SH-1	Seahorse	3/17/15
G. carolinianus	1503SH-2	Seahorse	3/17/15
G. carolinianus	1503SH-3	Seahorse	3/17/15
G. carolinianus	1503SH-7	Seahorse	3/17/15
G. carolinianus	1503SH-9	Seahorse	3/17/15
G. carolinianus	1503SH-10	Seahorse	3/17/15
G. carolinianus	1503SH-11	Seahorse	3/17/15
G. carolinianus	1503SH-12	Seahorse	3/17/15
G. carolinianus	1504BB-2	Benner Bay	4/13/15
G. carolinianus	1504BB-3	Benner Bay	4/13/15
G. carolinianus	1504BB-6	Benner Bay	4/13/15
G. carolinianus	1504BB-7	Benner Bay	4/13/15
G. carolinianus	1504BB-8	Benner Bay	4/13/15
G. carolinianus	1504BB-10	Benner Bay	4/13/15
G. carolinianus	1504BB-11	Benner Bay	4/13/15
G. carolinianus	1504BB-12	Benner Bay	4/13/15
G. carolinianus	1504BB-13	Benner Bay	4/13/15
G. carolinianus	1504BB-15	Benner Bay	4/13/15
G. carolinianus	1504BP-5	Black Point	4/13/15
G. carolinianus	1504BP-8	Black Point	4/13/15
G. carolinianus	1504BP-11	Black Point	4/13/15
G. carolinianus	1504BP-12	Black Point	4/13/15
G. carolinianus	1504FC-3	Flat Cay	4/13/15
G. carolinianus	1504FC-9	Flat Cay	4/13/15
G. carolinianus	1504FC-16	Flat Cay	4/13/15
G. carolinianus	1504SH-1	Seahorse	4/13/15
G. carolinianus	1504SH-5	Seahorse	4/13/15

Table B1: (continued)

G. carolinianus	1504SH-8	Seahorse	4/13/15
G. carolinianus	1505FC-4	Flat Cay	5/11/15
G. carolinianus	1505FC-7	Flat Cay	5/11/15
G. carolinianus	1505BB-1	Benner Bay	5/11/15
G. carolinianus	1505BB-2	Benner Bay	5/11/15
G. carolinianus	1505BB-5	Benner Bay	5/11/15
G. carolinianus	1505BB-8	Benner Bay	5/11/15
G. carolinianus	1505BB-10	Benner Bay	5/11/15
G. carolinianus	1505BB-11	Benner Bay	5/11/15
G. carolinianus	1505SH-5	Seahorse	5/11/15
G. carolinianus	1505SH-6	Seahorse	5/11/15
G. carolinianus	1505SH-7	Seahorse	5/11/15
G. carolinianus	1505SH-9	Seahorse	5/11/15
G. carolinianus	1505SH-11	Seahorse	5/11/15
G. carolinianus	1507SH-3	Seahorse	7/7/15
G. carolinianus	1507SH-6	Seahorse	7/7/15
G. carolinianus	1507SH-7	Seahorse	7/7/15
G. carolinianus	1507SH-9	Seahorse	7/7/15
G. carolinianus	1507SH-15	Seahorse	7/7/15
G. carolinianus	1507BB-3	Benner Bay	7/7/15
G. carolinianus	1507BB-5	Benner Bay	7/7/15
G. carolinianus	1507BB-6	Benner Bay	7/7/15
G. carolinianus	1507BB-8	Benner Bay	7/7/15
G. carolinianus	1507BB-15	Benner Bay	7/7/15
G. carolinianus	1507BB-16	Benner Bay	7/7/15
G. carolinianus	1507BB-17	Benner Bay	7/7/15
G. carolinianus	1507FC-3	Flat Cay	7/7/15
G. carolinianus	1507FC-6	Flat Cay	7/7/15
G. carolinianus	1507FC-9	Flat Cay	7/7/15
G. carolinianus	1507FC-11	Flat Cay	7/7/15
G. carolinianus	1507FC-13	Flat Cay	7/7/15
G. carolinianus	1507FC-14	Flat Cay	7/7/15
G. carolinianus	1507FC-15	Flat Cay	7/7/15
G. carolinianus	1507FC-16	Flat Cay	7/7/15
G. carolinianus	1507FC-17	Flat Cay	7/7/15
G. carolinianus	1507FC-20	Flat Cay	7/7/15
G. carolinianus	1507FC-23	Flat Cay	7/7/15

Table B1: (continued)

G. carolinianus	1507FC-27	Flat Cay	7/7/15
G. carolinianus	1507BP-1	Black Point	7/7/15
G. carolinianus	1507BP-5	Black Point	7/7/15
G. carolinianus	1507BP-6	Black Point	7/7/15
G. carolinianus	1507BP-7	Black Point	7/7/15
G. carolinianus	1507BP-9	Black Point	7/7/15
G. carpenteri	1310FC-2	Flat Cay	10/22/13
G. carpenteri	1310FC-6	Flat Cay	10/22/13
G. carpenteri	1311FC-1	Flat Cay	11/25/13
G. carpenteri	1311FC-3	Flat Cay	11/25/13
G. carpenteri	1311FC-5	Flat Cay	11/25/13
G. carpenteri	1311BP-2	Black Point	11/25/13
G. carpenteri	1311BP-3	Black Point	11/25/13
G. carpenteri	1311BP-5	Black Point	11/25/13
G. carpenteri	1311BP-6	Black Point	11/25/13
G. carpenteri	1311BP-7	Black Point	11/25/13
G. carpenteri	1401BP-5	Black Point	1/16/14
G. carpenteri	1402FC-8	Flat Cay	2/12/14
G. carpenteri	1402FC-10	Flat Cay	2/12/14
G. carpenteri	1402BP-3	Black Point	2/12/14
G. carpenteri	1402BP-5	Black Point	2/12/14
G. carpenteri	1402BP-12	Black Point	2/12/14
G. carpenteri	1402BB-6	Benner Bay	2/12/14
G. carpenteri	1402BB-12	Benner Bay	2/12/14
G. carpenteri	1403BB-2	Benner Bay	3/13/14
G. carpenteri	1409BP2-1	Black Point	9/2/14
G. carpenteri	1410FC-3	Flat Cay	10/22/14
G. carpenteri	1503FC-10	Flat Cay	3/17/15
G. carpenteri	1503BB-6	Benner Bay	3/17/15
G. carpenteri	1506BB-3	Benner Bay	6/2/15
G. carpenteri	1506BB-4	Benner Bay	6/2/15
G. carpenteri	1506BB-5	Benner Bay	6/2/15
G. ribotype 2	1311BB-1	Benner Bay	11/25/13
G. ribotype 2	1408BP-11	Black Point	8/6/14
G. silvae	1401SH-12	Seahorse	1/16/14
G. silvae	1402FC-2	Flat Cay	2/12/14
G. silvae	1402BP-7	Black Point	2/12/14

Table B1: (continued)

G. silvae	1402SH-11	Seahorse	2/12/14
G. silvae	1404BP-1	Black Point	4/15/14
G. silvae	1405FC-3	Flat Cay	5/14/14
G. silvae	1406BB-7	Benner Bay	6/10/14
G. silvae	1501SH-9	Seahorse	1/14/15
G. silvae	1503FC-3	Flat Cay	3/17/15
G. silvae	1503FC-9	Flat Cay	3/17/15
G. silvae	1504BP-1	Black Point	4/13/15
G. silvae	1504FC-11	Flat Cay	4/13/15
G. silvae	1504FC-12	Flat Cay	4/13/15
G. silvae	1504FC-14	Flat Cay	4/13/15
G. silvae	1504FC-15	Flat Cay	4/13/15
G. silvae	1504SH-7	Seahorse	4/13/15
G. silvae	1504SH-10	Seahorse	4/13/15
G. silvae	1504SH-12	Seahorse	4/13/15
G. silvae	1504SH-13	Seahorse	4/13/15
G. silvae	1505FC-3	Flat Cay	5/11/15
G. silvae	1505FC-5	Flat Cay	5/11/15
G. silvae	1505FC-10	Flat Cay	5/11/15
G. silvae	1505FC-11	Flat Cay	5/11/15
G. silvae	1505BB-6	Benner Bay	5/11/15
G. silvae	1505SH-1	Seahorse	5/11/15
G. silvae	1505SH-12	Seahorse	5/11/15
G. silvae	1505SH-14	Seahorse	5/11/15
G. silvae	1507FC-21	Flat Cay	7/7/15
TBD	1401SH-15	Seahorse	1/16/14
TBD	1401BB-4	Benner Bay	1/16/14
TBD	1401BB-10	Benner Bay	1/16/14
TBD	1402BP-8	Black Point	2/12/14
TBD	1402SH-1	Seahorse	2/12/14
TBD	1402BB-8	Benner Bay	2/12/14
TBD	1403BB-8	Benner Bay	3/13/14
TBD	1404BP2-7	Black Point	4/15/14
TBD	1404BB2-2	Benner Bay	4/15/14
TBD	1404BB2-4	Benner Bay	4/15/14
TBD	1405BB-5	Benner Bay	5/14/14
TBD	1405BB-6	Benner Bay	5/14/14

Table B1: (continued)

TBD	1405BB-7	Benner Bay	5/14/14
TBD	1409BP-3	Black Point	9/2/14
TBD	1409BP2-2	Black Point	9/2/14
TBD	1409BP2-5	Black Point	9/2/14
TBD	1409SH2-5	Seahorse	9/2/14
TBD	1409BB2-8	Benner Bay	9/2/14
TBD	1410FC-1	Flat Cay	10/22/14
TBD	1410FC-7	Flat Cay	10/22/14
TBD	1410SH-2	Seahorse	10/22/14
TBD	1410BP-15	Black Point	10/22/14
TBD	1503BB-7	Benner Bay	3/17/15

References

Adachi, R. and Y. Fukuyo. 1979. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et spec. nov. collected in a ciguatera-endemic area. *Bulletin of the Japanese Society of Scientific Fisheries*. 45(1): pp. 67-71.

Anderson, D.M., Y. Kaoru and A.W. White. 2000. Estimated Annual Economic Impacts from Harmful Algal Blooms (HABs) in the United States. Technical Report. 96 p.

Bagnis, R., S. Chanteau, E. Chungue, J.M. Hurtel, T. Yasumoto, and A. Inoue. 1980. Origins of ciguatera fish poisoning: A new dinoflagellate, *Gambierdiscus toxicus* Adachi and Fukuyo, definitely involved as a causal agent. *Toxicon*. 18: pp.199-208.

Band-Schmidt, C.J., E.L. Lilly, and D.M. Anderson. 2003. Identification of *Alexandrium affine* and *A. margalefii* (Dinophyceae) using DNA sequencing and LSU rDNA-based RFLP-PCR assays. *Phycologia*. 42 (3): pp. 261-268.

Caillaud, A., P. De la Iglesia, H.T. Darius, S. Pauillac, K. Aligizaki, S. Fraga, M. Chinain, and G. Diogene. 2010. Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. *Marine Drugs*. 8: pp. 1838–1907.

CDC - Centers for Disease Control and Prevention. 2009. Cluster of Ciguatera Fish Poisoning — North Carolina, 2007. *Morbidity and Mortality Weekly Report*. 58(11): pp. 283-285.

Chan, W.H., Y.L. Mak, J.J. Wu, L. Jin, W.H. Sit, J.C. Lam, Y.S. de Mitcheson, L.L. Chan, P.K. Lam, and B. Murphy. 2011. Spatial distribution of ciguateric fish in the Republic of Kiribati. *Chemosphere*. 84(1): pp. 117-23

Chinain, M., M.A. Faust, and S. Pauillac. 1999. Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov., and *G. polynesiensis* sp. Nov. *Journal of Phycology*. 35: pp. 1282–1296.

Chinain, M., H.T. Darius, A. Ung, M.T. Fouc, T. Revel, P. Cruchet, S. Pauillac, and D. Laurent. 2010. Ciguatera risk management in French Polynesia: The case study of Raivavae Island (Australes Archipelago). *Toxicon* 56: pp. 674–690.

Dickey, R.W and S.M. Plakas. 2010. Ciguatera: A public health perspective. *Toxicon* 56: pp. 123–136.

Erdner, D.L., M. Richlen, L.A.R. McCauley, and D.M. Anderson. 2011. Diversity and dynamics of a widespread bloom of the toxic dinoflagellate *Alexandrium fundyense*. *PLoS ONE* 6(7): e22965. doi:10.1371/journal.pone.0022965.

Faust, M.A. 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. *Journal of Phycology* 31: pp. 996–1003.

Fleming, L. E., D.G. Baden, J.A. Bean, R. Weisman, and D.G. Blythe. 1998. Seafood toxin diseases: issues in epidemiology and community outreach. In Reguera, B., J. Blanco, M.K. Fernandez, and T. Wyatt [Eds.] Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 245–248.

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

Fraga, S. and F. Rodríguez. 2014. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp. nov., a new potentially toxic epiphytic benthic dinoflagellate. *Protist*. 165: pp. 839–853.

Fraga, S., F. Rodríguez, P. Riobo, and I. Bravo. 2016. *Gambierdiscus balechii* sp. nov (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean). Harmful Algae. doi.org/10.1016/j.hal.2016.06.004

Friedman, M.A., L.E. Fleming, M. Fernandez, P. Bienfang, K. Schrank, R. Dickey, M.Y. Bottein, L. Backer, R. Ayyar, R. Weisman, S. Watkins, R. Granade and A. Reich. 2008. Ciguatera Fish Poisoning: Treatment, Prevention and Management. *Marine Drugs*. 6: pp. 456-479.

Galluzzi, L., E. Bertozzini, A. Penna, F. Perini, E. Garcés, M. Magnani. 2010. Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. *Journal of Applied Phycology*. 22(1): pp 1-9.

Guillou, L., E. Nézan, V. Cueff, E.E. Denn, M.A. Cambon-Bonavita, P. Gentien, and G. Barbier. 2002. Genetic diversity and molecular detection of three toxic dinoflagellate genera (*Alexandrium*, *Dinophysis*, and *Karenia*) from French Coasts. *Protist*. 153: pp. 223–238.

Gómez, F., D.J. Qiu, R.M. Lopes, and S.J. Lin. 2015. *Fukuyoa paulensis* gen. et sp nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). *PLoS ONE*. 10 (4):e0119676.

Halstead, B.W. 1978. Poisonous and venomous marine animals of the world. Princeton, N.J. Darwin Press. 283 p.

Hart, M.C., D.H. Green, E. Bresnan, and C.J. Bolch. 2007. Large subunit ribosomal RNA gene variation and sequence heterogeneity of *Dinophysis* (Dinophyceae) species from Scottish coastal waters. *Harmful Algae*. 6: pp. 271–287.

Heimann, K., and L. Sparrow. 2015. Ciguatera: Tropical Reef Fish Poisoning. In Handbook of Marine Microalgae: Biotechnology Advances. Se-Kwon Kim (Editor). Academic Press, 1 edition. 604 p.

Holmes, M.J. 1998. *Gambierdiscus yasumotoi* sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from southeastern Asia. *Journal of Phycology* 34: pp. 661–668.

Keller, M.D., R.C. Selvin, W. Claus, and R.R.L. Guillard. 1987. Media for the culture of oceanic ultraphytoplankton. *Journal of Phycology*. 23: pp. 633-638.

Kelly, A.M., C.C. Kohler and D.R. Tindall. 1992. Are crustaceans linked to the ciguatera food chain?. *Environmental Biology of Fishes*. 33: pp. 275 – 286.

Ki, J.S. and M.S. Han. 2007. Information characteristics of 12 divergent domains in complete large subunit rDNA sequences from the harmful dinoflagellate genus, *Alexandrium* (Dinophyceae). *Journal of Eukaryotic Microbiology*. 54: pp. 210–219.

Kuno, S, R. Kamikawa, S. Yoshimatsu, T. Sagara, S. Nishio, and Y. Sako. 2010. Genetic diversity of *Gambierdiscus* spp. (Gonyaulacales, Dinophyceae) in Japanese coastal areas. *Phycological Research*. 58 (1): pp. 44–52.

Lehane, L. and R.J. Lewis. 2000. Ciguatera: recent advances but the risk remains. *International Journal of Food Microbiology*. 61: pp. 91–125.

Lewis, R.J. and M.J. Holmes. 1993. Origin and transfer of toxins involved in ciguatera. *Comparative Biochemistry and Physiology – Part C*. 106C(3): pp. 615-628.

Lewis, R.J. 2006. Ciguatera: Australian perspectives on a global problem. *Toxicon*. 48: pp. 799–809.

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

Litaker, R.W., M.W. Vandersea, M.A. Faust, S.R. Kibler, A.W. Nau, W.C. Holland, M. Chinain, M.J. Holmes, and P.A. Tester. 2010. Global distribution of ciguatera causing dinoflagellates in the genus *Gambierdiscus*. *Toxicon*. 56: pp. 711–730.

Molinari, R.L., M. Spillane, I. Brooks, D. Atwood, and C. Duckett. 1981. Surface currents in the Caribbean Sea as deduced from Lagrangian observations. *Journal of Geophysical Research*. 86: pp. 6537-6542.

Moore, S. K., V.L. Trainer, N.J. Mantua, M.S. Parker, E.A. Laws, L.C. Backer and L.E. Fleming. 2008. Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environmental Health*. 7(2): S4.

Nishimura, T., S. Sato, W. Tawong, H. Sakanari, K. Uehara, M.M.R. Shah, S. Suda, T. Yasumoto, Y. Taira, H. Yamaguchi, and M. Adachi. 2013. Genetic diversity and distribution of the ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in coastal areas of Japan. *PLoS ONE*. 8 (4): e60882. doi:10.1371/journal.pone.0060882.

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

Parsons, M.L., K. Aligizaki, M.Y. Dechraoui, S. Fraga, S. Morton, A. Penna, and L. Rhodes. 2012. *Gambierdiscus* and *Ostreopsis*: reassessment of the state of knowledge of their taxonomy, geography, ecophysiology, and toxicology. *Harmful Algae*. 14: pp. 107–129.

Santoferrara, L.F., G.B. McManusa, and V.A. Alder. 2013. Utility of genetic markers and morphology for species discrimination within the order Tintinnida (Ciliophora, Spirotrichea). *Protist*. 164: pp. 24–36.

Scholin, C.A., M., Herzog, M., Sogin, and D.M. Anderson. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rRNA gene. *Journal of Phycology*. 30: pp. 999–1011.

Sonnenberg, R., A.W. Nolte, and D. Tautz. 2007. An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. *Frontiers in Zoology*. 4:6 doi:10.1186/1742-9994-4-6.

Tosteson, T.R. 1995. The diversity and origins of toxins in ciguatera fish poisoning. *Puerto Rico Health Sciences Journal*. 14: pp. 117–129.

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

Van Dolah, F.M. 2000. Marine Algal Toxins: Origins, Health Effects, and their Increased Occurrence. *Environmental Health Perspectives*. 108(1): pp. 133–141.

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

Wylezich, C., G. Nies, A.P. Mylnikov, D. Tautz, and H. Arndt. 2010. An evaluation of the use of the LSU rRNA D1-D5 domain for DNA based taxonomy of eukaryotic protists. *Protist*. 161: pp. 342–352.

Xu, Y., M.L. Richlen, S.L. Morton, Y.L. Mak, L.L. Chan, A. Tekiau, and D. Anderson. 2014. Distribution, abundance and diversity of *Gambierdiscus* spp. from a ciguatera-endemic area in Marakei, Republic of Kiribati. *Harmful Algae*. 34: pp. 56–68. doi: 10.1016/j.hal.2014.02.007.

Xu ,Y., ML. Richlen, J.D. Liefer, A. Robertson, D. Kulis, T.B. Smith, M.L. Parsons, D.M. Anderson. 2016. Influence of environmental variables on *Gambierdiscus* spp. (Dinophyceae) growth and distribution. *PLoS ONE*. 11(4): e0153197. doi:10.1371/journal.pone.0153197.

Yasumoto, T., I. Nakajima, R. Bagnis, and R. Adachi. 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. *Bulletin of the Japanese Society of Scientific Fisher*. 43(8): pp. 1021–1026.