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

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Highlights

- BsrI restriction enzyme digestion of the LSU rRNA D1-D2 region can distinguish the six *Gambierdiscus* species reported from the Caribbean

- This PCR-RFLP method successfully identified unknown *Gambierdiscus* species from field samples collected in the US Virgin Islands and Akumal beach -Mexico.
- PCR-RFLP is a rapid, inexpensive, and reliable method for identifying Caribbean *Gambierdiscus* species.

Abstract

The genus *Gambierdiscus* is a recognized group of marine epiphytic-benthic dinoflagellates that produce the toxins that cause ciguatera fish poisoning (CFP). To date, thirteen species and six 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 among species for research and monitoring purposes. *Gambierdiscus* species have very similar morphological characteristics and are difficult or impossible to distinguish using light microscopy. DNA sequencing has been an important tool in the definition of *Gambierdiscus* species, but it can be time-consuming and relatively expensive. To provide an alternative approach, a PCR-RFLP protocol was developed for efficient, rapid, and cost-effective identification of *Gambierdiscus* strains isolated from 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 also distinguishes them from four Pacific endemic species. This method was successfully used to type 465 clonal isolates of *Gambierdiscus* from the U.S. Virgin Islands and Akumal Beach - Mexico This BsrI PCR-RFLP method expands the tools available to researchers and managers engaged in monitoring activities and ecological studies.

Keywords: Harmful algae, benthic dinoflagellate, Bsr1 restriction enzyme, D1-D2 LSU, species identification

1. Introduction

Gambierdiscus (Adachi and Fukuyo, 1979) is a recognized genus of marine epiphytic-benthic dinoflagellates that produce gambiertoxins, precursors of the ciguatoxins that cause ciguatera fish poisoning (CFP). Globally, CFP is the most common foodborne illness associated with consumption of marine finfish (Yasumoto *et al*., 1977; Friedman *et al*., 2008). *Gambierdiscus* cells dwell on the surface of macroalgae, where they are grazed by herbivorous fish species, which are then eaten by carnivorous fishes, resulting in the propagation of toxins within the food web (Kelly *et al.*, 1992; Lewis and Holmes, 1993).

CFP causes significant public health and economic impacts and is expanding to non-endemic regions worldwide (Dickey and Plakas, 2010; Heimann and Sparrow, 2015). More than 400 commercially important fish species have been associated with ciguatoxins (Halstead, 1978; Lehane and Lewis, 2000; Van Dolah, 2000; Caillaud *et al*., 2010; Chan *et al*., 2011), thus CFP can have significant economic implications, especially to local fisheries in developing countries (Lehane and Lewis, 2000). Estimates of annual CFP cases worldwide vary from 50,000 – 500,000; the wide range reflects the high degree of underreporting of CFP (Fleming *et al*., 1998). In the Caribbean, Puerto Rico, and the U.S. Virgin Islands alone, it is suggested between 20,000 – 40,000 illnesses of ciguatera occur every year (Tosteson, 1995). CFP is limited to tropical and subtropical areas but in recent years it has been reported from new areas, due in part to increased tourism and trade of seafood from endemic areas (Dickey and Plakas, 2010; Vandersea *et al*., 2012). Also, climate change and anthropogenic impacts can contribute to 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, constituting a growing public health and socioeconomic threat.

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 more complex than first thought (Chinain *et al*., 1999; Lewis, 2006; Chinain *et al*., 2010). For many years *G. toxicus* Adachi and Fukuyo 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. To date there are 13 species (*G. australes*, *G. balechii*, *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. excentricus*, *G. lapillus*, *G. pacificus*, *G. polynesiensis*, *G. scabrosus*, *G. silvae*, *G. toxicus*) and six ribotypes (*G*. sp. ribotype 2, *G*. type 2 -6) of *Gambierdiscus* described using morphological characteristics and/or molecular tools (Faust, 1995; Chinain *et al*., 1999; Litaker *et al*., 2009; Kuno *et al*., 2010; Litaker *et al*., 2010; Fraga *et al*., 2011; Nishimura *et al*., 2013; Fraga and Rodríguez, 2014; Nishimura *et al*., 2014; Xu *et al*., 2014; Fraga *et al*., 2016; Kretzschmar *et al*., 2016). The species initially described as *G. yasumotoi* and *G. ruetzleri* showed enough morphological (globular form) and molecular differences (SSU and LSU phylogenies) to reclassify them in the new genus *Fukuyoa*. A third new species was recently described in this genus as *Fukuyoa paulensis* (Gómez *et al*. 2015).

Although the genus *Gambierdiscus* is widely distributed, the various *Gambierdiscus* species appear to be geographically restricted to particular ocean basins (Litaker *et al*., 2010). For example, several *Gambierdiscus* species have been isolated only from the Atlantic Ocean (*G. carolinianus*, *G. silvae* and *G.* ribotype 2), whereas others have been isolated only from the Pacific Ocean (*G. australes*, *G. pacificus*, *G. polynesiensis*, and *G. toxicus*); others like *G. carpenteri* and *G. caribaeus* have been found in both oceans. This shows that although the genus *Gambierdiscus* is widely distributed, species may be endemic to particular regions. Within a region, the latitudinal distribution of *Gambierdiscus* species may be increasing from tropical to temperate areas due to the effects of climate change (Moore *et al*., 2008). Studies in the Caribbean Sea and Gulf of Mexico have found multiple species of *Gambierdiscus* at a single location (Chinain *et al*., 1999; Litaker *et al*., 2009; Litaker *et al*., 2010), thus work is necessary at expanded temporal and spatial levels to better characterize the diversity and ecology of this dinoflagellate.

Molecular methods have been increasingly used for identification of *Gambierdiscus* species because morphological characteristics alone are not practical for routine identification. Overall morphology is very similar among species, and specific features can be phenotypically plastic, making it very difficult to distinguish species using light microscopy (Litaker *et al*., 2009). The identification of species based on morphological features is also 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 a species-specific molecular marker is available. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a simple molecular method that can differentiate species or ribotypes (Scholin and Anderson, 1994; Abe *et al*., 2013). Although it has become less common due to the development of high throughput sequencing, it continues to be a very useful method for fast and inexpensive screening of DNA sequence variation. Therefore, PCR-RFLP could provide a reliable, cheap, and rapid method to differentiate *Gambierdiscus* species in the GCR.

There is prior information that supports the use of specific DNA regions to identify species of *Gambierdiscus*. Small (SSU) and large (LSU) subunit rRNA gene sequences have shown their value as molecular markers in the identification of *Gambierdiscus* species (Chinain *et al.*, 1999; Litaker *et al*., 2009). Genetic distances based on the D1-D3 and D8-D10 regions show that the LSU rRNA provides sufficient resolution to discriminate between very closely related *Gambierdiscus* species, with the D1-D3 region exhibiting greater genetic distances (Chinain *et al*., 1999; Litaker *et al*., 2009; Fraga *et al*., 2014). Specifically, the D1-D2 hypervariable regions of the LSU rRNA gene have proven to be 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).

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. In this study, existing LSU rRNA gene sequences of *Gambierdiscus* spp. were used for *in silico* design of a PCR-RFLP method that could discriminate *Gambierdiscus* species. This assay was then validated using a set of independently identified DNA samples comprising different strains of eleven *Gambierdiscus* species. Lastly, it was used to type an extensive collection of *Gambierdiscus* isolates from the U.S. Virgin Islands and Mexican Coast in order to evaluate the utility of this PCR-RFLP method for identifying the different species present in this region. This method was shown to be accurate and robust, and thus should be applicable for analysis of isolates within this ocean region.

2. Materials and methods

2.1 Single cell isolation to establish cultured strains

Dinoflagellate samples were collected from the U.S. Virgin Islands in the Caribbean Sea and Mexican coast (Figure 1). Sampling sites were located in coastal waters south of the island of St. Thomas: Black Point (BP), Flat Cay (FC), Coculus Rock/Benner Bay (BB) and Seahorse (SH). Water and macroalgae samples were collected at these four sites every month from August 2013 to July 2015. Also, a sample was collected from Akumal Beach - Mexico on August 2013. Macroalgae were collected by SCUBA divers and placed into a one gallon plastic zipper bag with a small amount of surrounding seawater. The bag was stored in a cooler and returned to the lab for processing. There, the bag was agitated to dislodge the epiphyte cells from the macroalgae, and the contents were 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 University of Texas Marine Science Institute (UTMSI) at Port Aransas (Texas).

In the laboratory at UTMSI, 1 ml of sterile modified K Medium prepared with 0.2 µm-filtered and autoclaved natural seawater (Keller *et al*., 1987) was added to each container from the field. Samples were observed using a stereo microscope, and at least 12 single cells were isolated from each sample using microcapillary tubes. Every individual dinoflagellate cell isolated was sequentially transferred through five or more drops of sterile modified K medium to remove contaminants. Following these washing steps, the cell was then transferred to a single well of a 96 well cell culture plate containing 200 µl of modified K medium. Cells were incubated at 27 ºC under 12:12 light:dark conditions with approximately 90 µmol photons m^{-2} s^{-1} irradiance. 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 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 belonged to the *Gambierdiscus* genus.

2.2. DNA extraction from cultures

In almost all the tubes, cells were retained in the bottom of the tubes, but in some tubes the cells were found floating throughout the medium, surrounded by exopolymer. Cultures in exponential phase were harvested by taking cells from the bottom or water column of the culture tube where they were concentrated. 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 × g. The cells were washed twice with PBS buffer (BupH™ Phosphate Buffered Saline Pack – Thermo Fisher Scientific Inc) by resuspension in 1.5 ml of buffer followed by centrifugation, and the supernatant was removed and discarded. This process improved DNA extraction efficacy 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 resulting in a DNA extract volume of 200 µL, and stored at -20 ºC. The DNA extracts were quantified using a NanoVue Plus spectrophotometer (GE Healthcare, UK). DNA concentration ranged from 0.2 to 125.5 µg/ml.

2.3. 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 identifies 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. The input file contained LSU rRNA D1-D2 sequences from one strain of each species (Table 1). 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.

2.4. 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'-ACCCGCTGAATTTAAGCATA-3') and D2C (5'-CCTTGGTCCGTGTTTCAAGA-3') (Scholin *et al*., 1994). PCR amplification reactions (25 µl) contained ~5 ng template DNA, 1× 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 °C (after 1-2 minutes at 94 °C the cycle was paused to add the Taq), followed by 35 cycles of 30 seconds denaturing at 94 °C, 1 minute annealing at 50 °C, 2 minutes elongation at 72 °C, and a final elongation for 10 minutes at 72 °C. Successful amplification was verified using 3 µL of each PCR reaction mixed with 2 µL of loading dye containing GelRed™ (1:300 dilution) nucleic acid gel stain (Biotium, Hayward, CA, USA), checked by 0.8% agarose gel electrophoresis (0.5× TBE) and visualized under UV light.

2.5. 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 NEBuffer 3.1 (10X), 18 ul water, 0.3 µl of BsrI restriction enzyme, and 4 µl of PCR product. Each sample was digested by incubation at 65 °C for 60 minutes followed by inactivation at 80 °C for 20 minutes. From each digested sample, 6 µl of digest were mixed with 2 µl of loading dye with GelRed™ (1:300 dilution) nucleic acid gel stain (Biotium, Hayward, CA, USA), and resolved on a 2.0% agarose gel with 0.5× TBE buffer. Gel images of the RFLP band patterns were recorded using a digital camera (FOTO/Analyst® Express Systems, Foto/UV 26, Fotodyne Inc. Hartland, WI, USA). Logger Pro software (Vernier Software & Technology, Beaverton, OR, USA) was used to determine the fragment size of each band.

The assay was first validated 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* (Table 2). Genomic DNA from some species and strains was scarce, in which case D1-D2 PCR products were used. Also, no samples of *G. excentricus*, *G. lapillus*, and *G*. sp. type 2 – 6 were available, so these species were not tested.

To evaluate the applicability of this method using samples from the field, 472 DNA extracts obtained from strains cultured in the laboratory were analyzed. The details of these samples, established from collections in the U.S. Virgin Islands and Akumal beach - Mexico, is presented in Supplementary Table S1.

3. Results

3.1 *In silico* prediction

Analysis of *Gambierdiscus* DNA sequences predicted that the BsrI restriction enzyme could distinguish the six *Gambierdiscus* species reported from the GCR (*G. belizeanus*, *G. caribaeus, G. carpenteri, G. carolinianus, G. silvae, Gambierdiscus* sp. ribotype 2). *In silico* restriction digest using this enzyme generated a specific number of bands and fragment sizes that were unique for each species (Table 1). The virtual digests are not, however, able to differentiate all *Gambierdiscus* species worldwide using the single BsrI enzyme. The fragments predicted for *G. toxicus* and *G. pacificus* were very similar in size; while they are not identical they may be practically indistinguishable given the resolving power of agarose gels. This is also the case for *G. excentricus* and *G. caribaeus*. For seven of the known species or ribotypes (*G. balechii, G. lapillus,* and *G.* type 2-6), no D1-D2 sequences were available, thus we could not predict the RFLP performance with these species.

3.2 Assay validation

The *in silico* RFLP predictions were first validated using a collection of known DNAs from eleven *Gambierdiscus* species whose 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 of nearly the same size. For nearly all species, the predicted digest patterns matched those observed from the known DNA samples. In one case, that of *G. belizeanus,* the number and size of the bands predicted from the *in silico* digestion was markedly different from the profile predicted by the virtual BsrI digestion (Table 1). For this species the virtual digest predicted two bands, but six bands were observed in the gel from the actual PCR-RFLP digestion. For five species, *G. carolinianus*, *G. caribaeus*, *G. australes*, *G. pacificus* and *G. toxicus,* the *in silico* digestion predicted a band of small size that was very blurry and not easily observed in the gel. For four species, *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 visually 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 (Table 1). For the six species that have been found previously in the Caribbean, the assay produces clearly distinguishable profiles (Figure 2). Digestion with BsrI produced two bands in *G. carolinianus*, *G. silvae, G.* ribotype2 and *G. carpenteri*, four bands in *G. caribaeus*, and six bands in *G. belizeanus,* with different band sizes in each species.

A few similarities in banding patterns were observed, however these instances were between Pacific and Caribbean species and did not prevent the differentiation of the species present in the GCR from Pacific *Gambierdiscus* species (Figure 3). *Gambierdiscus pacificus* and *G. toxicus* showed the same number and size of bands, but did not share this pattern with any Caribbean species. Profiles of *G. carolinianus* and *G. polynesiensis* showed a similar profile with a single band, but with a small size difference. *Gambierdiscus carolinianus* showed a second blurry band around 600 bp, which distinguishes this species from *G. polynesiensis*. Within each species the banding patterns were consistent among multiple strains evaluated (Figures 4 through 7).

3.3 Identification of unknown isolates

After validation, the utility of the BsrI RFLP method was tested using cultured strains established from field collections from the U.S. Virgin Islands and Akumal beach - Mexico. A total of 1161 single cells were isolated, resulting in 495 cultured strains. This corresponded to a 42% culture success rate with this single cell isolation method. In the 96 well cell culture plate, 70% of isolated cells grew sufficiently that they were transferred to a small volume (5 ml) culture, and 62% of those were transferred to standard volume (25 ml) culture. DNA was extracted from all of the 495 strains for RFLP analysis. A small minority of the samples (4.6% or 23 strains) did not produce successful PCR products despite multiple attempts. The remaining 472 strains were successfully typed by the BsrI PCR-RFLP method.

The RFLP patterns of each of the 472 strains matched one of the six species that have been reported from the GCR: *G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. belizeanus*, *G. silvae* or *G*. ribotype 2. None of the RFLP patterns matched those species considered to be restricted to the Pacific: *G. australes*, *G. pacificus*, *G. polynesiensis*, *G. scabrosus*, and *G. toxicus*. No novel banding patterns were observed. In the culture collection, *G. carolinianus* and *G. caribaeus* were the most common species with 41.1% and 34.9% of strains, respectively (Figure 8). Only two strains of *G.* ribotype2 (0.4%) were found. Other species represented a low percentage of strains identified: *G. belizeanus* (10.8%), *G. carpenteri* (6.8%), and *G. silvae* (5.9%). Only two species were identified in the Akumal beach – Mexico with six strains of *G. carpenteri* and one strain of *G. caribaeus*.

4. Discussion

The goal of this effort was to develop an easy-to-use method for routine identification of *Gambierdiscus* species. The BsrI PCR-RFLP assay described here enables consistent and reliable discrimination of the six species of *Gambierdiscus* reported from the Caribbean. This method is faster and less expensive than sequencing of the rRNA genes, as it can be completed in one day and does not incur the time and expense of sequence analysis. It provides an alternative or complement to light microscopy, which can be challenging due to morphological similarities between the species and the need for taxonomic expertise in identification. 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 472 U.S. Virgin Islands and Akumal beach isolates typed by RFLP were all identified as one of the six species reported from the Caribbean.

The BsrI RFLP assay is most applicable for the study of *Gambierdiscus* species in the Caribbean because the species described from this region produce markedly different banding patterns. These are consistent, repeatable and, most importantly, visually distinct. 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, the overall banding patterns are sufficiently different that small size variation in individual bands 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 – two bands, widely spaced – even though the fragment sizes are slightly smaller than in the other strains. The BsrI banding patterns of the Caribbean *Gambierdiscus* species can easily be recognized by eye, for example the two widely spaced bands of *G. silvae* vs. the two closely spaced bands of *G. carpenteri*, in contrast to the ladder-like pattern of *G. belizeanus* or the single long fragment of *G. carolinianus*.

The BsrI RFLP method can also aid in the identification of Pacific *Gambierdiscus* strains, with limitations. Similarity in banding patterns within one pair of Pacific species and between one pair of Pacific and Caribbean species limits its use for discriminating all species of *Gambierdiscus*. The BsrI enzyme produces identical RFLP patterns for the Pacific species *G. toxicus* and *G. pacificus*. Both of these are known from the Pacific only, thus their separation would require the use of another method, for example the three-enzyme RFLP method described by Lyu *et al*. (In press). Phylogenetic analysis of LSU rRNA gene sequences shows that this species pair exhibits a low level of genetic differentiation, which here was reflected in a lack of restriction enzyme polymorphism (Chinain *et al*., 1999; Litaker *et al*., 2009; 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: 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. *Gambierdiscus 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 41 bp) 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 bp.

In the present study, the PCR-RFLP method was successfully applied to identify unknown *Gambierdiscus* species from field samples taken in the U.S. Virgin Islands and Akumal beach – Mexico. This methodology allows identification of species using molecular information from recent studies from this genus, and represents an important advance in the study of the *Gambierdiscus* in the GCR. This method provides an easy and efficient way to identify isolates cultured for specific purposes, e.g. physiological or population genetic studies. While two species, *G. caribaeus and G. carolinianus*, dominated our collection from the Virgin Islands, this is not a direct reflection of species abundance in the sampling area, as it is influenced by the efficiency of single cell isolation and culturing for various species. Similarly, with a low number of isolates from Akumal beach – Mexico, it is not expected that *G. carpenteri* and *G. caribaeus* are the only species found in this area. Direct determination of the abundance and distribution of *Gambierdiscus* cells and species in the field therefore necessitates the development of other methods that can be used directly without cell cultivation. The culture methods used in this study 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.

For many studies, it is desirable to quantify the abundance and distribution of *Gambierdiscus* cells and species in the field, and this will require development of methods that can be used directly without cell cultivation and therefore culture bias. However, fast and inexpensive RFLP methods can be applied for approximating community composition in natural samples. The LSU D1-D2 region can be easily amplified by PCR from single *Gambierdiscus* cells (Richlen and Barber, 2005) followed by RFLP analysis to identify the species. In this way, the isolation and RFLP analysis of multiple single cells from a field sample could provide a first estimate of the diversity and relative abundance of species in an area.

The new PCR-RFLP method developed in this study is a practical, useful, quick, inexpensive, 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.

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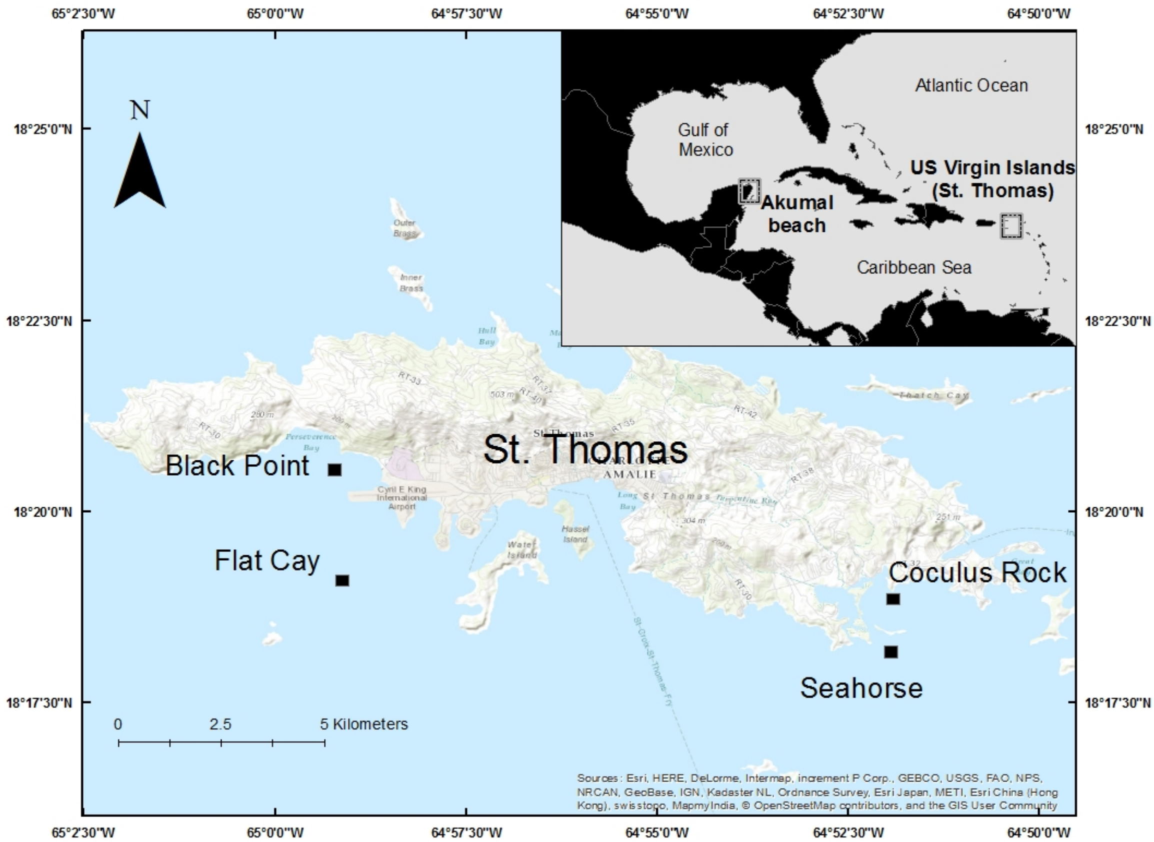

Figure 1. Study area with sampling locations in St. Thomas, U.S. Virgin Islands in the Caribbean Sea and Akumal beach -Mexico.

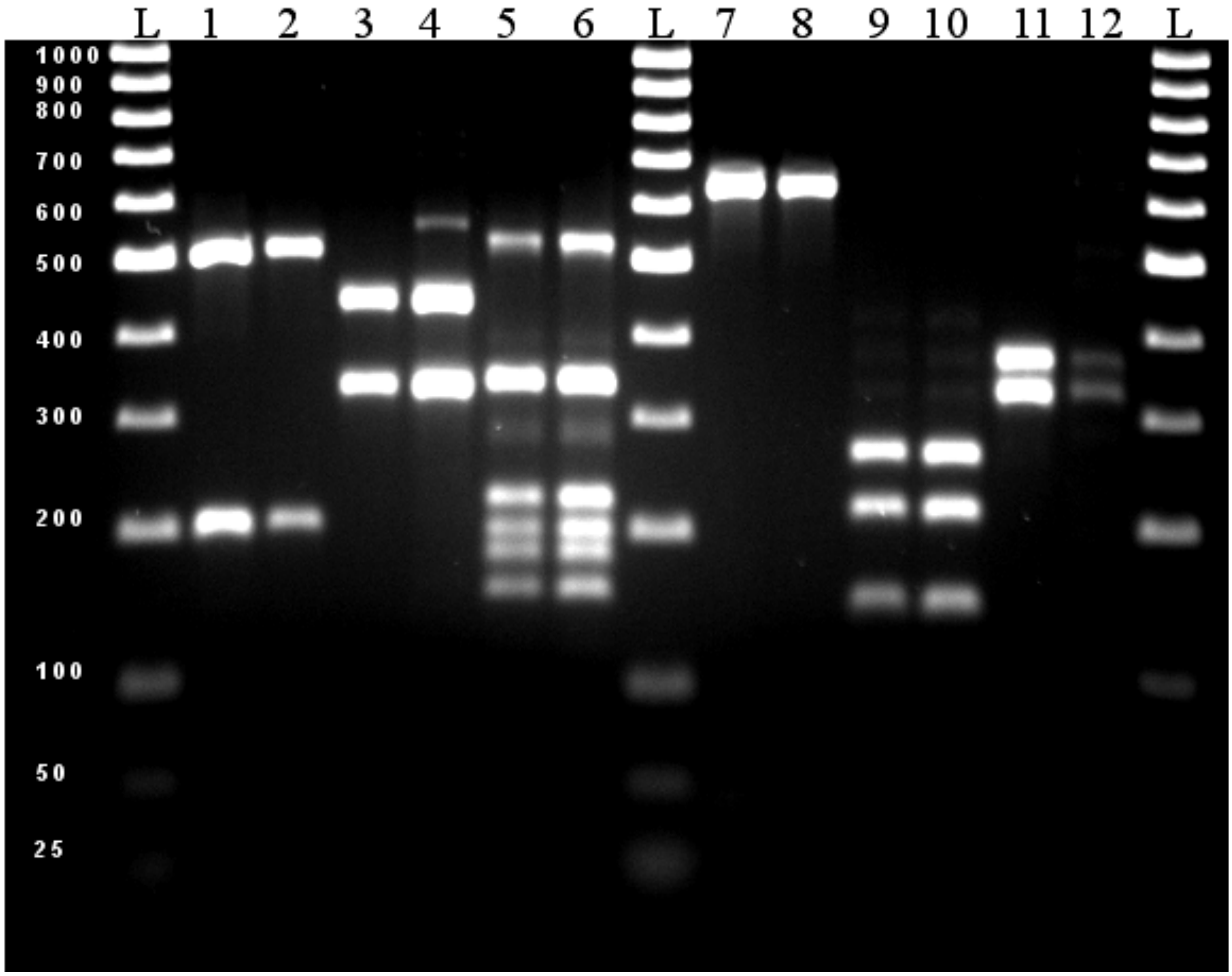

Figure 2. BsrI-digested PCR products of the LSU rRNA D1-D2 region from the six species of *Gambierdiscus* found in the Greater Caribbean Region. Lane 1-2 *G. silvae* (strains silv2 and silv4), lane 3-4 *G.* ribotype2 (strains ribo2-2 and ribo2-4), lane 5-6 *G.belizeanus* (strains beli5 and beli7), lane 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 2.

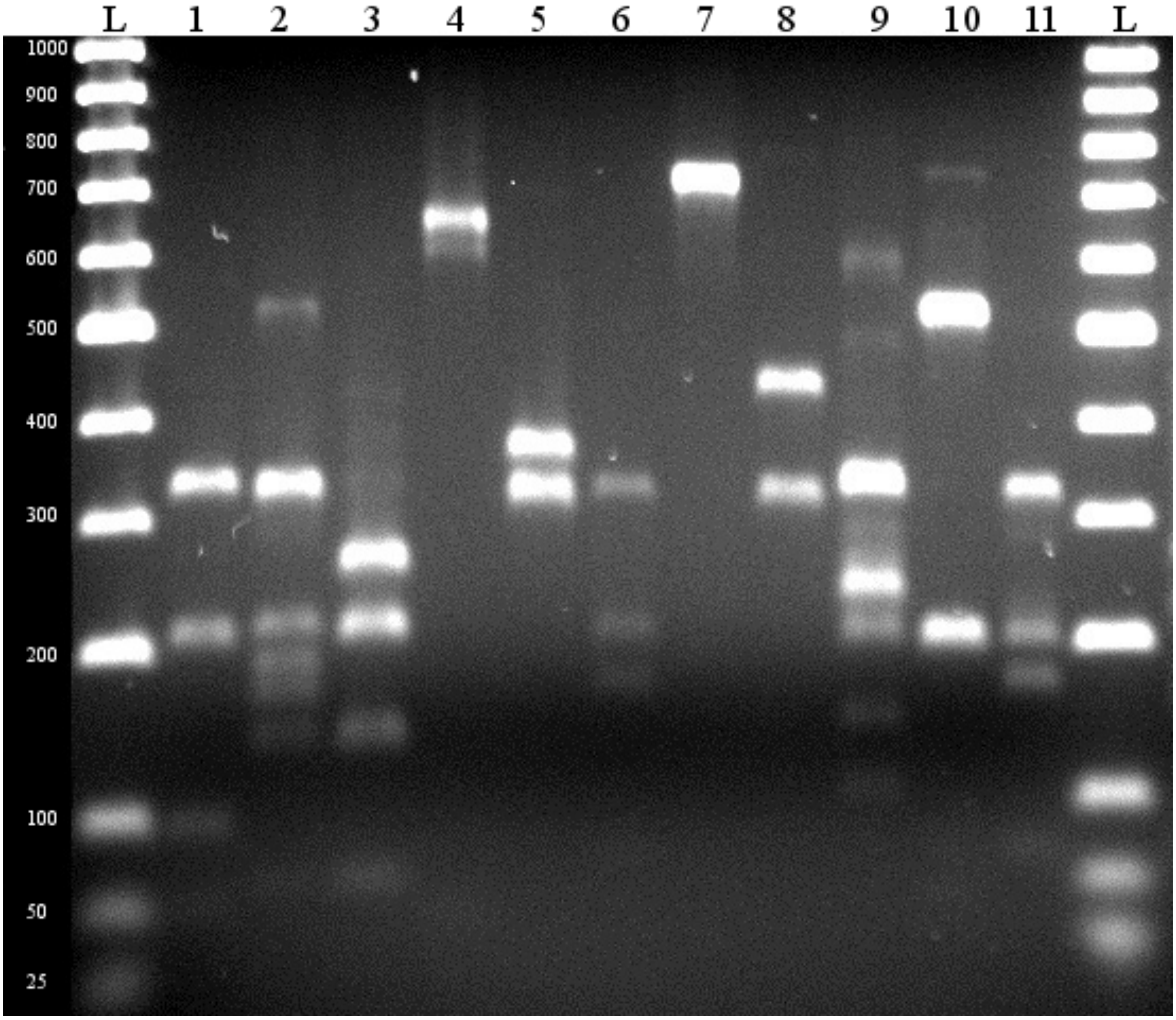

Figure 3. Restriction patterns of BsrI-digested PCR products of the LSU D1-D2 region from eleven *Gambierdiscus* species. 1. *G. australes* (aust2), 2. *G. belizeanus* (beli5), 3. *G. caribaeus* (cari3), 4. *G. carolinianus* (caro2), 5. *G. carpenteri* (carp2), 6. *G. pacificus* (paci1), 7. *G. polynesiensis* (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 2.

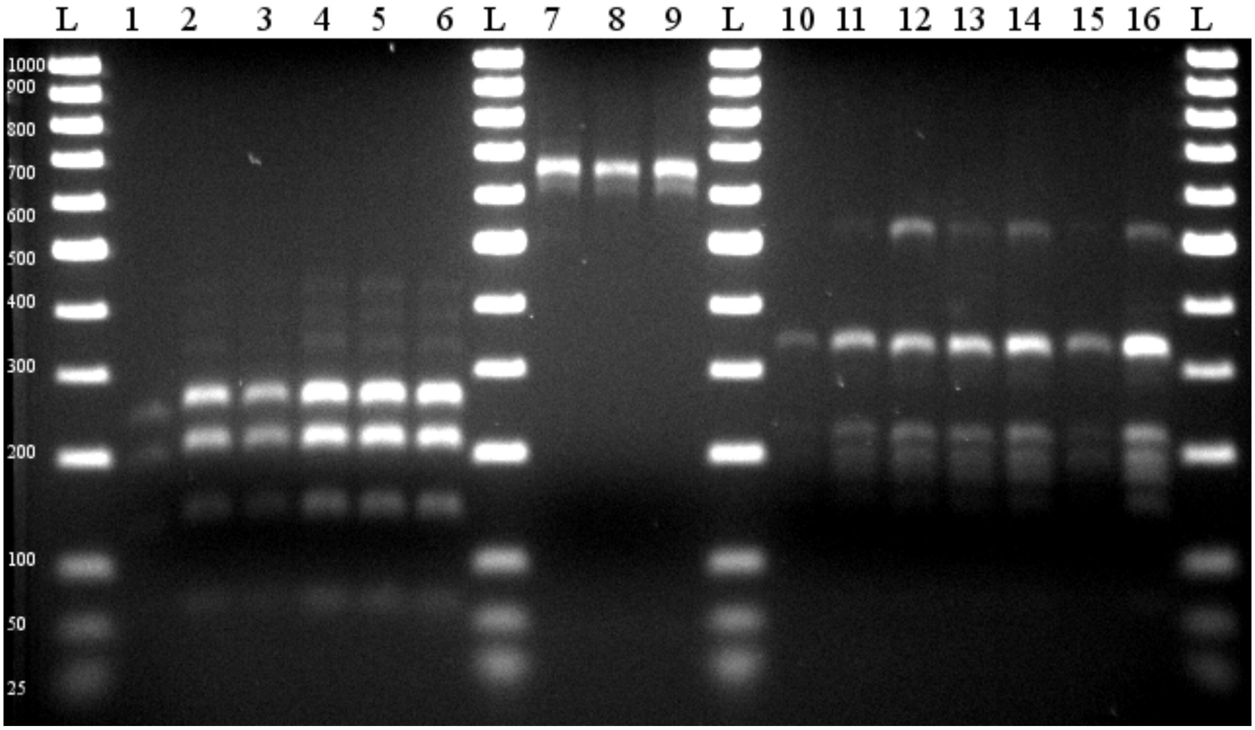

Figure 4. Restriction patterns of BsrI-digested PCR of the LSU D1-D2 region from multiple strains of three *Gambierdiscus* species. Lane 1-6 *G. caribaeus* (strains cari2, cari3, cari5, cari1, cari6 and cari4), Lane 7-8 *G. carolinianus* (strains caro1, caro2 and caro3), Lane 10-16 *G. belizeanus* (strains beli1, beli2, beli3, beli4, beli5, beli6 and beli7); Lane L 100bp PCR DNA Ladder. Strain designations are in Table 2.

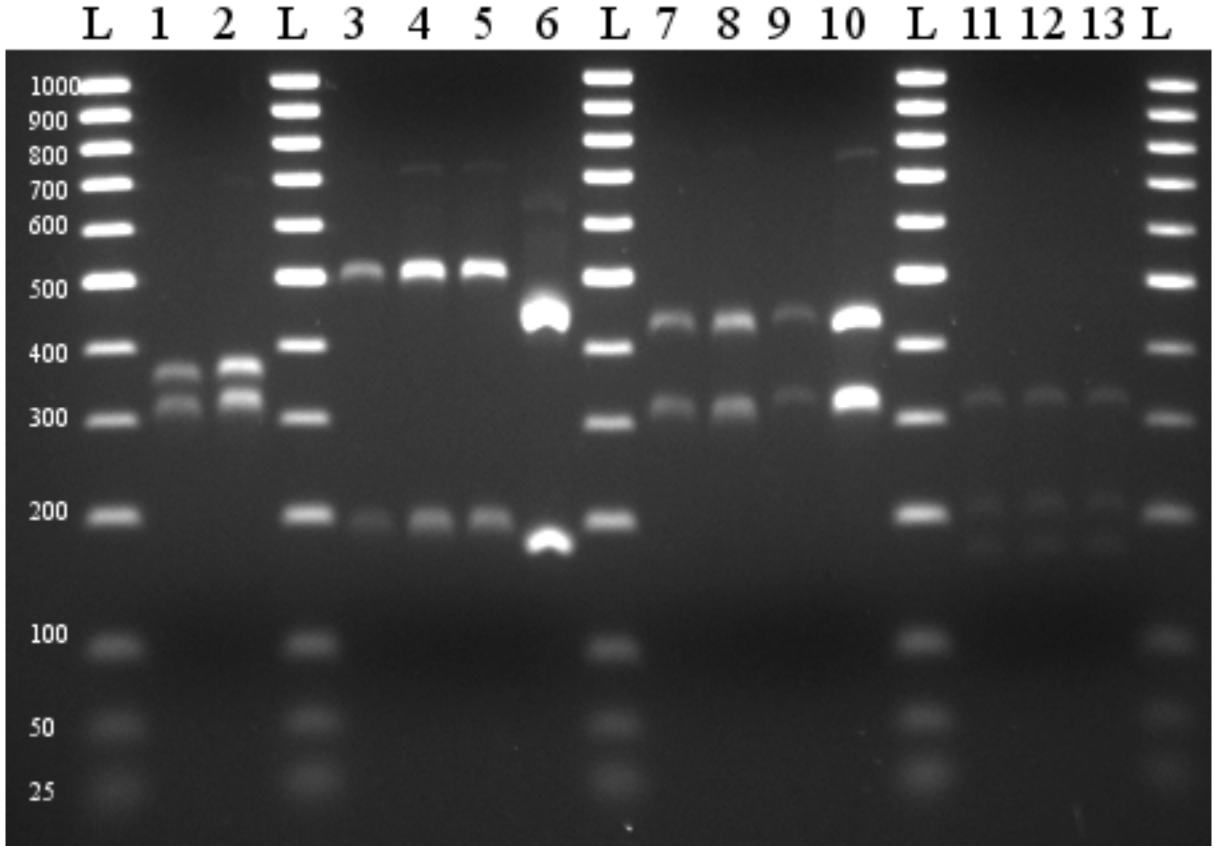

Figure 5. Restriction patterns of BsrI-digested PCR of the LSU D1-D2 region from multiple strains of four *Gambierdiscus* species. Lane 1-2 *G. carpenteri* (strains carp1 and carp2), Lane 3-6 *G. silvae* (strains silv1, silv2, silv3 and silv4), Lane 7-10 *G.* ribotype 2 (strains ribo2-1, ribo2-2, ribo2-3,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

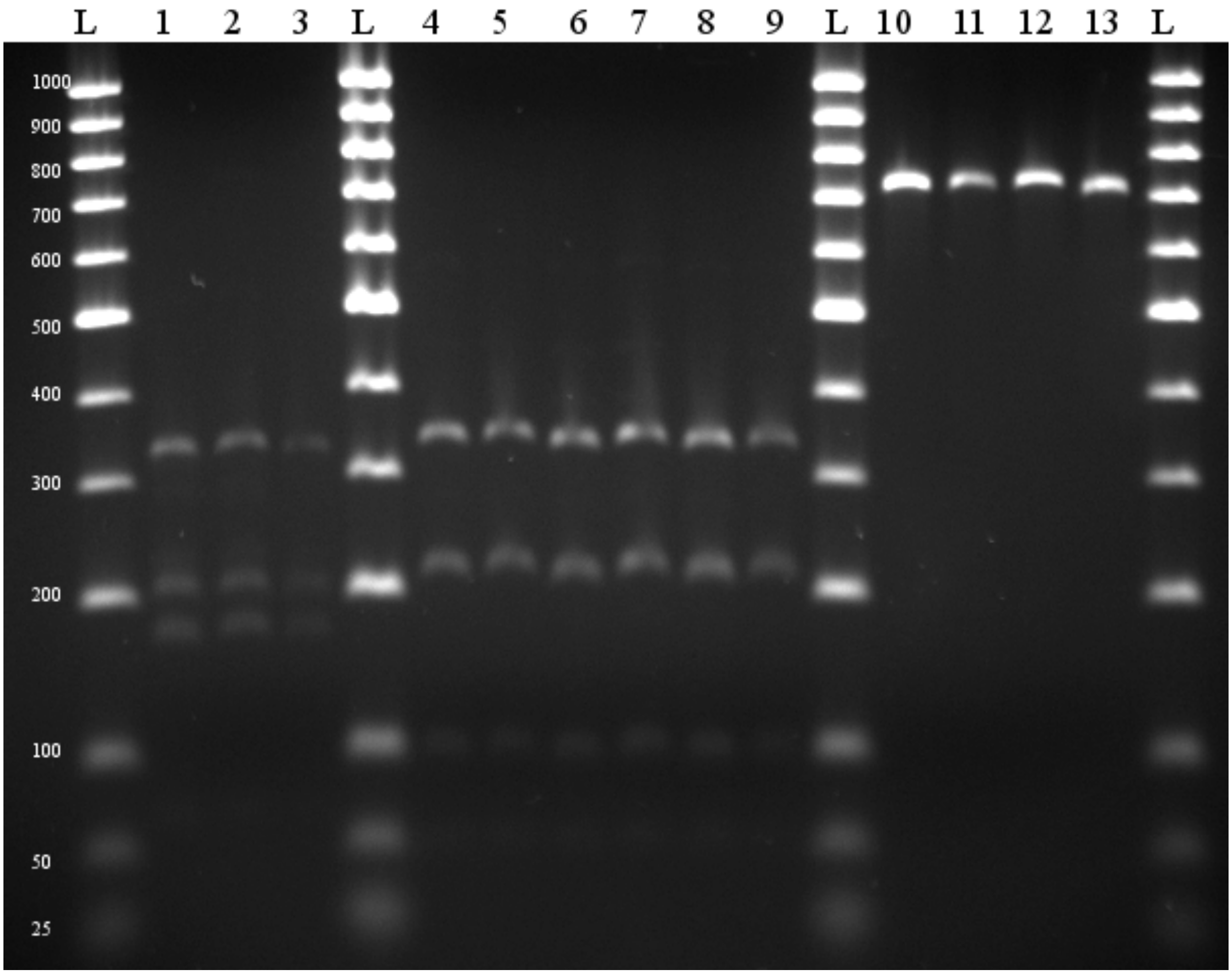

Figure 6. Restriction patterns of BsrI-digested PCR of the LSU D1-D2 region from four *Gambierdiscus* species. Lane 1-3 *G. toxicus* (strains toxi1, toxi2 and toxi3), Lane 4-9 *G. australes* (strains aust1, aust2, aust3, aust4, aust5, and aust6) Lane 10-13 *G. polynesiensis* (strains poly1, poly2, poly3 and poly4), Lane L 100bp PCR DNA Ladder. Strain designations are in Table 2.

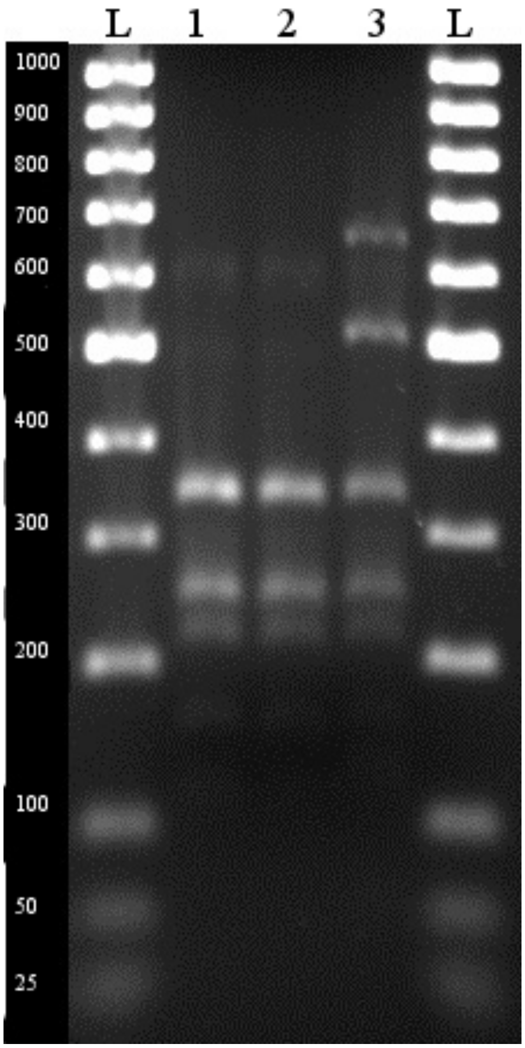

Figure 7. Restriction patterns from BsrI-digested PCR of the LSU D1-D2 region of *G. scabrosus.* Lane 1-3 strains scab1, scab2 and scab3. Lane L 100bp PCR DNA Ladder. Strain designations are in Table 2.

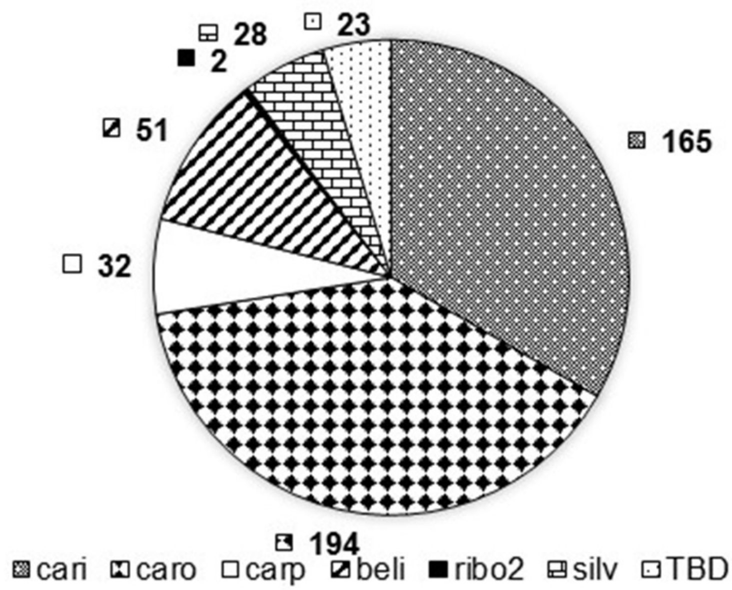

Figure 8. Number of strains of each species from the U.S.Virgin Islands and Akumal beach -Mexico identified with the PCR-RFLP method. cari: *G. caribaeus*, caro: *G. carolinianus*, carp: *G. carpenteri*, beli: *G. belizeanus*, ribo2: *G*. ribotype 2, silv: *G. silvae* and TBD: to be determined.

Table 1. Predicted and observed fragment sizes (bp) for each of the *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	Strain used to predict the fragment (GenBank Accession number)	Predicted Fragment size	Actual Fragment size
G. belizeanus	**Caribbean**	**NOAA5_1_8 (EF202948.1)**	**425 321**	**515 318 216 196 183 162**
G. caribaeus	**Caribbean Pacific**	**NOAA11_4 (EF202937.1)**	**260 210 145 58**	**260 220 160 60**
G. carolinianus	**Caribbean**	**NOAA6_1_6 (EF202975.1)**	**647 41**	**650 50**
G. carpenteri	**Caribbean Pacific**	**NOAA1_5 (EF202939.1)**	**355 316**	**355 310**
G. silvae	**Caribbean Atlantic**	**FC May 10-9 (WHOI)**	**485 181**	**515 210**
***G.* ribotype 2**	**Caribbean**	**SH Dec 10-10 (WHOI)**	**404 304**	**420 310**
G. excentricus	Atlantic	VGO 792 (JF303071.1)	251 230 160 57	No Data
G. australes	Pacific	RAV92_1 (EF202970.1)	332 219 98 48	320 210 125 50
G. pacificus	Pacific	HO91_4 (EF202947.1)	320 211 176	317 215 185

			69	70
G. polynesiensis	Pacific	TB92_3 (EF202982.1)	703 (No Cut Sites)	730
G. scabrosus	Pacific	GM10 (AB604964.1)	303 239 197 20	325 243 215 170 143
G. toxicus	Pacific	TUR_4 (EF202961.1)	320 202 175 67	320 210 190 70

Table 2. Strains of *Gambierdiscus* spp. used for assay validation, and results of RFLP analysis.

Isolates	Geographic Origin	Abbreviation	Molecular species ID
BB Apr 11-11	St. Thomas, USVI	Cari1	*G. caribaeus*
BP Aug 08	St. Thomas, USVI	Cari2	*G. caribaeus*
HGB7	Florida Keys, FL, USA	Cari3	*G. caribaeus*
LKH4	Florida Keys, FL, USA	Cari4	*G. caribaeus*
Tenn10	Florida Keys, FL, USA	Cari5	*G. caribaeus*
CR May 10-12	St. Thomas, USVI	Cari6	*G. caribaeus*
1401BP2	St. Thomas, USVI	Cari7	Not sequenced
1309FC4-7	St. Thomas, USVI	Cari8	Not sequenced
GHCG2-C6	San Salvador, Bahamas	Caro1	*G. carolinianus*
GHCG2-A6	San Salvador, Bahamas	Caro2	*G. carolinianus*
GHCG2-B8	San Salvador, Bahamas	Caro3	*G. carolinianus*
Cheeca1	Florida Keys, FL, USA	Caro4	*G. carolinianus*
CCMP399	St. Barthelemy Island	Beli1	*G. belizeanus*
FC Dec 10-13	St. Thomas, USVI	Beli2	*G. belizeanus*
BP Apr 11-7	St. Thomas, USVI	Beli3	*G. belizeanus*
BP Mar 10-18	St. Thomas, USVI	Beli4	*G. belizeanus*
BP Mar 10-25	St. Thomas, USVI	Beli5	*G. belizeanus*
BP Mar 10-31	St. Thomas, USVI	Beli6	*G. belizeanus*
BP Mar 10-7	St. Thomas, USVI	Beli7	*G. belizeanus*
MUR4	Moruroa, French Polynesia	Paci1	*G. pacificus*
HO91	Otepa, Hao, French Polynesia	Paci2	*G. pacificus*
TubET1	Mahu, Tubuai, French Polynesia	Paci3	*G. pacificus*
BP Apr 11-6	St. Thomas, USVI	Ribo2-1	*G.* sp. ribotype 2
SH Dec 10-10	St. Thomas, USVI	Ribo2-2	*G.* sp. ribotype 2
SH Dec 10-12	St. Thomas, USVI	Ribo2-3	*G.* sp. ribotype 2
TRL29	Florida Keys, FL,	Ribo2-4	*G.* sp. ribotype 2

	USA		
KML1	Florida Keys, FL, USA	Carp1	*G. carpenteri*
TPH12	Florida Keys, FL, USA	Carp2	*G. carpenteri*
1506BB3	St. Thomas, USVI	Carp3	Not sequenced
1402FC8	St. Thomas, USVI	Carp4	Not sequenced
PO		Aust1	*G. australes*
RAV1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Aust2	*G. australes*
G3/93		Aust3	*G. australes*
S080911-1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Aust4	*G. australes*
ISC5G		Aust5	*G. australes*
I080606-1		Aust6	*G. australes*
Rai1		Poly1	*G. polynesiensis*
Rik8		Poly2	*G. polynesiensis*
RG92		Poly3	*G. polynesiensis*
TB-92	Tubuai, French Polynesia	Poly4	*G. polynesiensis*
GTT1		Toxi1	*G. toxicus*
RIK13		Toxi2	*G. toxicus*
HIT-0	Tahiti, French Polynesia	Toxi3	*G. toxicus*
BP Mar 10-23	St. Thomas, USVI	Silv1	*G. silvae*
FC May 10-9	St. Thomas, USVI	Silv2	*G. silvae*
SH Apr 11-1	St. Thomas, USVI	Silv 3	*G. silvae*
TRL23	Florida Keys, FL, USA	Silv 4	*G. silvae*
KW070922-1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Scab1	*G. scabrosus*

KW070922-2	Kashiwa-jima Island, Otsuki, Kochi, Japan	Scab2	*G. scabrosus*
TO80908-1	Kashiwa-jima Island, Otsuki, Kochi, Japan	Scab3	*G. scabrosus*

Appendix A. Supplementary data.

Supplementary table S1. Strains of Gambierdiscus species isolated from ST. Thomas – US Virgin Islands and Akumal beach -Mexico 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	Coculus Rock	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	Coculus Rock	4/15/14
G. belizeanus	1404BB2-8	Coculus Rock	4/15/14
G. belizeanus	1404BB2-11	Coculus Rock	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	Coculus Rock	9/2/14
G. belizeanus	1409BB2-6	Coculus Rock	9/2/14
G. belizeanus	1409BB2-7	Coculus Rock	9/2/14
G. belizeanus	1409BB2-9	Coculus Rock	9/2/14
G. belizeanus	1409BB2-10	Coculus Rock	9/2/14
G. belizeanus	1501SH-10	Seahorse	1/14/15
G. belizeanus	1504BB-9	Coculus Rock	4/13/15
G. belizeanus	1504FC-1	Flat Cay	4/13/15
G. belizeanus	1507BB-12	Coculus Rock	7/7/15
G. belizeanus	1507BB-18	Coculus Rock	7/7/15
G. belizeanus	1507BB-19	Coculus Rock	7/7/15
G. belizeanus	1507BB-21	Coculus Rock	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	AKU-03	Akumal Beach - Mexico	8/26/13
G. caribaeus	1308SH2-1	Seahorse	8/26/13
G. caribaeus	1308BB1-10	Coculus Rock	8/26/13
G. caribaeus	1308SH2-3	Seahorse	8/26/13
G. caribaeus	1308SH2-5	Seahorse	8/26/13
G. caribaeus	1308BB1-4	Coculus Rock	8/26/13

G. caribaeus	1308BB1-7	Coculus Rock	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	Coculus Rock	10/22/13
G. caribaeus	1310BB-3	Coculus Rock	10/22/13
G. caribaeus	1310BB-5	Coculus Rock	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
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	Coculus Rock	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
G. caribaeus	1406FC-2	Flat Cay	6/10/14
G. caribaeus	1406BB-2	Coculus Rock	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	Coculus Rock	7/8/14
G. caribaeus	1407BB-5	Coculus Rock	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	Coculus	8/6/14

		Rock	
G. caribaeus	1409BP-4	Black Point	9/2/14
G. caribaeus	1409SH-1	Seahorse	9/2/14
G. caribaeus	1409BB-2	Coculus Rock	9/2/14
G. caribaeus	1409BB-4	Coculus Rock	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	Coculus Rock	9/2/14
G. caribaeus	1410FC-2	Flat Cay	10/22/14
G. caribaeus	1410FC-4	Flat Cay	10/22/14
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
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	Coculus Rock	3/17/15
G. caribaeus	1503BB-9	Coculus Rock	3/17/15
G. caribaeus	1503BB-10	Coculus Rock	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	Coculus Rock	4/13/15
G. caribaeus	1504BB-14	Coculus Rock	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	Coculus Rock	5/11/15
G. caribaeus	1505BB-4	Coculus Rock	5/11/15
G. caribaeus	1505BB-12	Coculus Rock	5/11/15
G. caribaeus	1505BB-14	Coculus Rock	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	Coculus Rock	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
G. carolinianus	1401SH-16	Seahorse	1/16/14
G. carolinianus	1401BB-1	Coculus Rock	1/16/14
G. carolinianus	1401BB-2	Coculus Rock	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	Coculus Rock	2/12/14

G. carolinianus	1402BB-5	Coculus Rock	2/12/14
G. carolinianus	1402BB-14	Coculus Rock	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	Coculus Rock	3/13/14
G. carolinianus	1403BB-4	Coculus Rock	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	Coculus Rock	4/15/14
G. carolinianus	1404BB2-5	Coculus Rock	4/15/14
G. carolinianus	1405BP-5	Black Point	5/14/14
G. carolinianus	1405BP-6	Black Point	5/14/14
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	Coculus Rock	6/10/14
G. carolinianus	1406BB-4	Coculus Rock	6/10/14
G. carolinianus	1406BB-6	Coculus Rock	6/10/14
G. carolinianus	1406BB-11	Coculus Rock	6/10/14
G. carolinianus	1406BB-13	Coculus Rock	6/10/14
G. carolinianus	1407SH-3	Seahorse	7/8/14
G. carolinianus	1407BB-7	Coculus Rock	7/8/14
G. carolinianus	1407BB-10	Coculus Rock	7/8/14
G. carolinianus	1407BB-12	Coculus Rock	7/8/14

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	Coculus Rock	8/6/14
G. carolinianus	1408BB-4	Coculus Rock	8/6/14
G. carolinianus	1409SH2-11	Seahorse	9/2/14
G. carolinianus	1409BB2-4	Coculus Rock	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	Coculus Rock	1/14/15
G. carolinianus	1501BB-2	Coculus Rock	1/14/15
G. carolinianus	1501BB-4	Coculus Rock	1/14/15
G. carolinianus	1501BB-5	Coculus Rock	1/14/15
G. carolinianus	1501BB-6	Coculus Rock	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
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	Coculus Rock	3/17/15
G. carolinianus	1503BB-2	Coculus Rock	3/17/15
G. carolinianus	1503BB-3	Coculus Rock	3/17/15
G. carolinianus	1503BB-4	Coculus Rock	3/17/15
G. carolinianus	1503BB-5	Coculus Rock	3/17/15
G. carolinianus	1503BB-11	Coculus Rock	3/17/15
G. carolinianus	1503BB-12	Coculus Rock	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	Coculus Rock	4/13/15
G. carolinianus	1504BB-3	Coculus Rock	4/13/15
G. carolinianus	1504BB-6	Coculus Rock	4/13/15
G. carolinianus	1504BB-7	Coculus Rock	4/13/15
G. carolinianus	1504BB-8	Coculus Rock	4/13/15
G. carolinianus	1504BB-10	Coculus	4/13/15

		Rock	
G. carolinianus	1504BB-11	Coculus Rock	4/13/15
G. carolinianus	1504BB-12	Coculus Rock	4/13/15
G. carolinianus	1504BB-13	Coculus Rock	4/13/15
G. carolinianus	1504BB-15	Coculus Rock	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
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	Coculus Rock	5/11/15
G. carolinianus	1505BB-2	Coculus Rock	5/11/15
G. carolinianus	1505BB-5	Coculus Rock	5/11/15
G. carolinianus	1505BB-8	Coculus Rock	5/11/15
G. carolinianus	1505BB-10	Coculus Rock	5/11/15
G. carolinianus	1505BB-11	Coculus Rock	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	Coculus Rock	7/7/15
G. carolinianus	1507BB-5	Coculus Rock	7/7/15
G. carolinianus	1507BB-6	Coculus Rock	7/7/15
G. carolinianus	1507BB-8	Coculus Rock	7/7/15
G. carolinianus	1507BB-15	Coculus Rock	7/7/15
G. carolinianus	1507BB-16	Coculus Rock	7/7/15
G. carolinianus	1507BB-17	Coculus Rock	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
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	AKU-01	Akumal Beach - Mexico	8/26/13
G. carpenteri	AKU-05	Akumal Beach - Mexico	8/26/13

G. carpenteri	AKU-07	Akumal Beach - Mexico	8/26/13
G. carpenteri	AKU-08	Akumal Beach - Mexico	8/26/13
G. carpenteri	AKU-10	Akumal Beach - Mexico	8/26/13
G. carpenteri	AKU-13	Akumal Beach - Mexico	8/26/13
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	Coculus Rock	2/12/14
G. carpenteri	1402BB-12	Coculus Rock	2/12/14
G. carpenteri	1403BB-2	Coculus Rock	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	Coculus Rock	3/17/15
G. carpenteri	1506BB-3	Coculus	6/2/15

		Rock	
G. carpenteri	1506BB-4	Coculus Rock	6/2/15
G. carpenteri	1506BB-5	Coculus Rock	6/2/15
G. ribotype 2	1311BB-1	Coculus Rock	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
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	Coculus Rock	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	Coculus Rock	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	Coculus Rock	1/16/14
TBD	1401BB-10	Coculus Rock	1/16/14
TBD	1402BP-8	Black Point	2/12/14
TBD	1402SH-1	Seahorse	2/12/14
TBD	1402BB-8	Coculus Rock	2/12/14
TBD	1403BB-8	Coculus Rock	3/13/14
TBD	1404BP2-7	Black Point	4/15/14
TBD	1404BB2-2	Coculus Rock	4/15/14
TBD	1404BB2-4	Coculus Rock	4/15/14
TBD	1405BB-5	Coculus Rock	5/14/14
TBD	1405BB-6	Coculus Rock	5/14/14
TBD	1405BB-7	Coculus Rock	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	Coculus Rock	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	Coculus Rock	3/17/15