Genetic Diversity and Distribution of the Ciguatera-Causing Dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in Coastal Areas of Japan

Tomohiro Nishimura1,2, Shinya Sato3,4, Wittaya Tawong1,2, Hiroshi Sakanari1, Keita Uehara1, Md Mahfuzur Rahman Shah5,6, Shoichiro Suda5, Takeshi Yasumoto7, Yohsuke Taira8, Haruo Yamaguchi1, Masao Adachi1*

1 Faculty of Agriculture, Kochi University, Nankoku, Kochi, Japan, 2 The United Graduate School of Agricultural Sciences, Matsuyama, Ehime University, Ehime, Japan, 3 Royal Botanic Garden Edinburgh, Edinburgh, United Kingdom, 4 Cardiff University, Cardiff, Wales, United Kingdom, 5 Faculty of Science, University of the Ryukyus, Nakagami District, Okinawa, Japan, 6 College of Ocean Science, Jeju National University, Jeju, South Korea, 7 National Research Institute of Fisheries Science, Yokohama, Kanagawa, Japan, 8 Okinawa Institute of Science and Technology Evolutionary Systems Biology Unit, Kunigami District, Okinawa, Japan

**Abstract**

**Background:** The marine epiphytic dinoflagellate genus *Gambierdiscus* produce toxins that cause ciguatera fish poisoning (CFP): one of the most significant seafood-borne illnesses associated with fish consumption worldwide. So far, occurrences of CFP incidents in Japan have been mainly reported in subtropical areas. A previous phylogeographic study of Japanese *Gambierdiscus* revealed the existence of two distinct phylotypes: *Gambierdiscus* sp. type 1 from subtropical and *Gambierdiscus* sp. type 2 from temperate areas. However, details of the genetic diversity and distribution for Japanese *Gambierdiscus* are still unclear, because a comprehensive investigation has not been conducted yet.

**Methods/Principal Finding:** A total of 248 strains were examined from samples mainly collected from western and southern coastal areas of Japan during 2006–2011. The SSU rDNA, the LSU rDNA D8–D10 and the ITS region were selected as genetic markers and phylogenetic analyses were conducted. The genetic diversity of Japanese *Gambierdiscus* was high since five species/phylotypes were detected: including two reported phylotypes (Gambierdiscus sp. type 1 and Gambierdiscus sp. type 2), two species of Gambierdiscus (*G. australis* and G. cf. *yasumotoi*) and a hitherto unreported phylotype Gambierdiscus sp. type 3. The distributions of type 3 and G. cf. *yasumotoi* were restricted to the temperate and the subtropical area, respectively. On the other hand, type 1, type 2 and *G. australis* occurred from the subtropical to the temperate area, with a tendency that type 1 and *G. australis* were dominant in the subtropical area, whereas type 2 was dominant in the temperate area. By using mouse bioassay, type 1, type 3 and *G. australis* exhibited mouse toxicities.

**Conclusions/Significance:** This study revealed a surprising diversity of Japanese *Gambierdiscus* and the distribution of five species/phylotypes displayed clear geographical patterns in Japanese coastal areas. The SSU rDNA and the LSU rDNA D8–D10 as genetic markers are recommended for further use.

---

**Introduction**

Ciguatera fish poisoning (CFP) is one of the most significant marine food-borne illnesses caused by eating reef fish, it affects 25,000–500,000 people annually around the world; and this is endemic throughout the tropical and subtropical Pacific, Indian Ocean and the tropical Caribbean Sea [1,2,3,4,5,6,7]. Common symptoms of this syndrome include gastrointestinal, neurological and cardiovascular disturbances [5,6,8]. In some cases, the neurological disturbance usually resolves within weeks of onset, although some symptoms related to the nervous system may persist for months or years [5,8].

Occurrence of CFP incidents was rather rare in Japan. Until the 1980’s CFP incidents were restricted within the subtropical area (Okinawa region) [9]; however, in recent years CFP incidents have increasingly been reported not only from the subtropical area but also from the temperate area (including the main islands of Japan, Honshu, Shikoku and Kyushu regions) [10,11,12]. Thus, the public health problem is becoming more serious along Japanese coastal areas.

In the late 1970’s, a marine epiphytic dinoflagellate was discovered and identified as a possible producer of the toxins responsible for CFP [13] and later described as *Gambierdiscus toxicus* Adachi and Fukuyo by morphological observations [14]. *G. toxicus* could produce ciguatoxins (CTXs) which are transferred to
herbivorous and carnivorous fish via food chain [13]. The genus Gambierdiscus may also produce other toxins such as maitotoxins (MTXs), gambierol and gambieric acid [15].

Routine identification of Gambierdiscus species using light microscopy (LM) and scanning electron microscopy (SEM) is very difficult even for trained taxonomists because species descriptions are based on subtle differences in the thecal plate morphology [14,15,16,17,19,20] that are mostly quantitative and largely overlap. Some early studies described three species of Gambierdiscus based on SEM observations, that is, G. toxicus [14], G. belzebatus [16], G. yasumotoi [17].

Molecular information has enabled us to assess the phylogenetic relationship more objectively, and Gambierdiscus species are by no means excluded from this technical advance [e.g. [20]]. All the phylogenetic analyses of Gambierdiscus conducted so far have been inferred from nuclear-encoded ribosomal RNA gene (rDNA) and have shown that each species is partitioned into unique clades that correspond with its morphological features [15,18,20]. In 1999, Chinain et al. [18] described G. pacificus, G. australis and G. polyenesensis by morphological and phylogenetic analysis of the D8–D10 region of the nuclear large subunit (LSU) rDNA (LSU rDNA D8–D10) for the first study introducing molecular phylogenetic technique for the classification of Gambierdiscus species. In 2009, Litaker et al. [20] described four new species, G. caribaeus, G. carolinianus, G. carperti and G. ruetzleri, based on morphological characteristics together with molecular phylogenies using the nuclear small subunit (SSU) rDNA, the D1–D3 region of the LSU rDNA (LSU rDNA D1–D3) and the LSU rDNA D8–D10. They also performed a sequence analysis of the ITS1-5.8 S rDNA-ITS2 (ITS region) in order to discriminate G. ruetzleri from its sister species G. yasumotoi as both species were the most closely related of the Gambierdiscus species [20]. Furthermore, an amended description of G. toxicus with the designation of an epitype specimen was provided [20]. In their subsequent paper in 2010, two phylogenies (putative species), called Gambierdiscus ribotype 1 and Gambierdiscus ribotype 2, were reported based on the LSU rDNA D8–D10 phylogeny from the Atlantic [21]. Coincidently in 2010 from Japanese coastal areas in the Pacific, a further two putative allopatric phytophages were detected with the SSU rDNA and the ITS region: Gambierdiscus sp. type 1 from the subtropical and Gambierdiscus sp. type 2 from the temperate areas [22]. Recently in 2011, Fraga et al. [15] described G. excentricus by morphological and phylogenetic analyses of the LSU rDNA D1–D3 and the LSU rDNA D8–D10. In 2012 from Jeju Island, Korea, Pacific, Jeong et al. [23] reported the existence of a possibly cryptic species of ‘G. caribaeus’ by using phylogenics of the SSU rDNA, the LSU rDNA D1–D3 and the LSU rDNA D8–D10, and morphological observations by SEM.

Gambierdiscus spp. distribute globally from the Atlantic (including Gulf of Mexico and Caribbean Sea) to the Pacific (Australia to Hawaii) [21,24] and ref therein) as well as the Bay of Bengal, India [25], Cau Island, Vietnam [26], Indonesia [27], La Union, Philippines [28], Langkawi Island, Redang Island, Port Dickson, Kota Kinabalu and Sipadan Island, Malaysia [29,30,31], Jeju Island, Korea [23,32] and Taiwan [33]. Japanese coasts have also been investigated over the past few decades and revealed that Gambierdiscus spp. distribute widely from subtropical to temperate areas [11,22,34,35,36,37,38]. Additionally, in previous studies in Japan, ‘G. toxicus’ has been found from the temperate to subtropical areas in Japanese coasts based on morphologically observations [11,34,35,36,37].

In the present study, we apply the phylogeographic approach with the genetic marker used by Litaker et al. [20] (the SSU rDNA, the LSU rDNA D8–D10 and the ITS region) to have a more complete picture of the diversity of Japanese Gambierdiscus spp. We particularly placed emphasis on revealing the distribution of each species and each phyotype, which we define here as a monophyletic entity based on a molecular phylogeny that has not been taxonomically described but has accumulated an equivalent degree of sequence divergence with other described species. In addition, toxicity was tested with mouse bioassay for a representative clone selected from each phyotype.

Results

Phylogenetic Analyses of the SSU and the LSU D8–D10

The topologies of Bayesian inference (BI) tree for the SSU rDNA and the LSU rDNA D8–D10 of Gambierdiscus spp. including species/phytophotypes from Japan (Figs. 1 and 2) were almost the same and the same as that of the Maximum likelihood (ML) tree for the LSU rDNA D8–D10 of Gambierdiscus spp. including Japanese species/phytophotypes (Fig. S1). The topologies of the former BI trees were almost the same as that of BI tree of Gambierdiscus spp. for the LSU rDNA reported by Litaker et al. [20] and those for the LSU rDNA D8–D10 reported by Fraga et al. [15] and Litaker et al. [20,21]. Discrepancies between the ML and the BI trees were only found in the positions of Gambierdiscus ribotype 2 and G. belzebatus (Fig. 2 and Fig. S1). Therefore we used the BI trees for discussion of both the SSU rDNA and the LSU rDNA D8–D10 phylogenies.

Both BI phylogenetic trees showed that, each Gambierdiscus species/phytophotype was clearly separated into five genetically distinct clades I–V and each clade received the highest nodal supports (Figs. 1 and 2), i.e. Bayesian posterior probability (pp)/bootstrap value (bt) = (1.00/100) obtained by BI analysis and ML analysis, respectively. In the SSU rDNA tree (Fig. 1), the major clades I–V branched off one by one in a ladder-like fashion, viz. (I|II|III|IV|V)). The topology LSU rDNA D8–D10 tree (Fig. 2) was substantially the same as that of the SSU rDNA tree recovering five major clades, except for the position of clade II that was sister to clade IV, viz., (I|II|III|IV|V|)).

Clade I was comprised of G. yasumotoi (0.68/77 for the LSU rDNA D8–D10 tree), G. ruetzleri (1.00/77 and 1.00/99 for the SSU rDNA tree and for the LSU rDNA D8–D10 tree, respectively) and two Japanese strains IR4 G and Go3 (0.58/39 for the SSU rDNA tree, no the LSU rDNA D8–D10 sequence for the latter strain) established in the present study (green letters), the both strains were provisionally named as Gambierdiscus cf. yasumotoi in the present study based on its gross morphology and sequence analysis of the ITS region (see below, genetic distance section).

Clade II, in the SSU rDNA tree (Fig. 1), had G. carperti (1.00/97) which diverged at first followed by the second divergence of G. carperti (1.00/100) and Gambierdiscus sp. type 2 (1.00/100), whereas, in the LSU rDNA D8–D10 tree (Fig. 2), the first diverged species/phytophotype was Gambierdiscus sp. type 2 (1.00/100) and the second ones were G. carperti (1.00/87) and G. caribaeus (1.00/95). Type 2, in the SSU rDNA tree (Fig. 1), was solely comprised of Japanese strains including 14 strains established in the present study (blue letters) and two strains (black letters) by Kuno et al. [22].

Clade III had G. carolinianus (1.00/100 and 1.00/100) which was sister to G. polyenesensis (1.00/94 and 1.00/97), Gambierdiscus ribotype 1 (1.00/99 for the LSU rDNA D8–D10 tree) whose SSU rDNA sequences were unavailable and Gambierdiscus sp. type 5 (1.00/100 and 1.00/100) including two strains (W19 G and WW11 G) established in the present study (orange letters).

Clade IV was exclusively consisted of strains of G. australis (1.00/100 and 1.00/100), in the SSU rDNA tree (Fig. 1), among which
seven strains were collected in the present study (purple letters) and one strain (black letter) by Litaker et al. [20], and *G. excentricus* (1.00/100) whose SSU rDNA sequences were unavailable in the LSU rDNA D8–D10 tree (Figs. 1 and 2).

Clade V was the most diverse clade, containing five robust phylotypes/species - *Gambierdiscus* ribotype 2 (1.00/100) diverged at first followed by the second divergence of *G. belizeanus* (1.00/100) in the SSU rDNA tree (Fig. 1), whereas the first diverged
Phylotype was *G. belizeanus* and the second one was *Gambierdiscus* ribotype 2 in the LSU rDNA D8–D10 tree (Fig. 2). Subsequently, *Gambierdiscus* sp. type 1 (1.00/100 and 1.00/93), in the SSU rDNA tree (Fig. 1), contained 16 strains collected in the present study (red letters) and two strains (black letters) by Kuno et al. [22], branched off that was sister to *G. pacificus* (1.00/95 and 0.82/88) and *G. toxicus* (1.00/100 and 1.00/97).

Within type 1, in the SSU rDNA tree, there was a distinct phylogenetic structure corresponding to the geographic origin of the strains, i.e. a moderately supported branch (1.00/84) indicated

Figure 2. Bayesian inference (BI) phylogeny of the D8–D10 region of the LSU rDNA of *Gambierdiscus* species/phylotypes. Nodal supports are of Bayesian posterior probability (pp) and Bootstrap (bt) values obtained by BI analysis and Maximum likelihood (ML) analysis, respectively. Nodes with strong supports (pp/bt = 1.00/100) are shown as thick lines. For sequences obtained via cloning, a variant ID, starting with C, is shown followed by strain ID (i.e. T080908_1_C1). Sequences obtained in the present study are indicated in color. doi:10.1371/journal.pone.0060882.g002

Diversity and Distribution of *Gambierdiscus* in JPN

PLOS ONE | www.plosone.org 4 April 2013 | Volume 8 | Issue 4 | e60882
by an asterisk in Fig. 1 was consisted of the temperate strains, whereas the others in the type 1 were all of subtropical origin.

As a consequence, the genetic diversity of Japanese Gambierdiscus revealed in the present study was high since we detected five species/phylogenies including two species of Gambierdiscus (G. australis and G. cf. yasumotoi), two reported phylotypes (Gambierdiscus sp. type 1 and Gambierdiscus sp. type 2 [22]) and a hitherto unreported phylotype Gambierdiscus sp. type 3.

Genetic Distances

For revealing whether Japanese phylotypes (Gambierdiscus sp. type 1, type 2 and type 3) had species level divergence or not, we calculated p distance of the both genes using uncorrected genetic distance (UGD) model among Gambierdiscus species. In case of the SSU rDNA, the mean p distance of within-species and that of between-species were 0.003±0.002 and 0.139±0.042, respectively (Table 1). The latter value was significantly different from the former value (t-test, P<0.01). The minimum p distance of between-species (0.004) was found between G. yasumotoi and G. ruetzleri (Table 1 and Table S1). For the LSU rDNA D8–D10, the mean p distance of within-species and between-species were 0.002±0.002 and 0.121±0.036, respectively (Table S2). Between-species divergence of the LSU rDNA D8–D10 was significantly different from that of the SSU rDNA (t-test, P<0.01). The minimum p distance of between-species (0.007) was found between G. yasumotoi and G. ruetzleri (Tables S2 and S3). The mean p distance of between-species of the SSU rDNA was significantly different from that of the LSU rDNA (t-test, P<0.05).

We calculated the minimum p distances of the two genes between each Japanese phylotype (type 1, type 2 and type 3) and another species. Then, we compared those of the minimum values and the minimum value obtained from between-species. As a result, we revealed that the minimum values obtained between each Japanese phylotype and another species, e.g. type 1 vs. G. pacificus (0.040 and 0.018 for the SSU rDNA and the LSU rDNA D8–D10, respectively), type 2 vs. G. carpenteri (0.020 for the SSU rDNA), type 2 vs. G. carpenteri (0.025 for the LSU rDNA D8–D10) and type 3 vs. G. polyedron (0.033 and 0.024), were larger than that of the pair G. yasumotoi vs. G. ruetzleri (0.004 and 0.007), which was the minimum value of between-species (Table 1 and Table S2).

The SSU rDNA and the LSU rDNA D8–D10 phylogenies indicated that our strains IR4G and Go3 (LSU rDNA D8–D10 sequence was unavailable for the latter strain) belonged to the clade I along with G. yasumotoi and G. ruetzleri (Figs. 1 and 2). A further test of their within-clade relationship by means of the uncorrected p distance of the ITS region among the strain IR4G (GenBank accession number: AB765925), G. yasumotoi and G. ruetzleri showed that IR4G was more closely related to G. yasumotoi than G. ruetzleri, i.e. the p distance between IR4G and G. yasumotoi was 0.0252 (12 sites in 476 bp), whereas between IR4G and G. ruetzleri was 0.0479 (23 sites in 480 bp). Therefore, Japanese 2 strains (IR4G and Go3) were tentatively identified as G. cf. yasumotoi by referring to the indicative p distance of the species limit on the ITS region according to Litaker et al. [39].

Distribution of Gambierdiscus in Coastal Areas of Japan

We collected macroalgal samples from 73 stations (50 and 23 originated from the temperate area and the subtropical area, respectively) in Japan during 2006–2011 (Fig. 3 and Table S4). In total 248 unialgal strains of Gambierdiscus (197 and 51 originated from the temperate area and the subtropical area, respectively) were successfully established from 24 stations (11 and 13 originated from the temperate area and the subtropical area, respectively) (yellow circles in Fig. 3). In the present study, five Gambierdiscus species/phylogenies (G. australis, G. cf. yasumotoi, Gambierdiscus sp. type 1, Gambierdiscus sp. type 2 and Gambierdiscus sp. type 3) were found in the western and the southern coastal zones.

Table 1. Summary of genetic distances of the SSU rDNA over the p distance calculated by uncorrected genetic distance (UGD) model among and within 66 consensus sequences (=66 strains; each consensus sequence was constructed with cloned sequences and/or directly sequence of each strain) of Gambierdiscus species/phylogenies, and other protists.

| Protist          | Genus           | bp* | n** | Min. | Max. | Mean | SD   | Reference*** |
|------------------|-----------------|-----|-----|------|------|------|------|-------------|
| Dinoflagellate   | Gambierdiscus   | 1759| 10  | 0.000| 0.005| 0.003| 0.002| This study  |
|                  | within-species  | 1759| 10  | 0.004| 0.180| 0.139| 0.042| This study  |
|                  | between-species | 1759| 11  | 0.040| 0.172| 0.121| 0.050| This study  |
|                  | type 1 vs. another species | 1759| 11  | 0.028| 0.163| 0.129| 0.051| This study  |
|                  | type 2 vs. another species | 1759| 11  | 0.033| 0.168| 0.140| 0.042| This study  |
|                  | type 3 vs. another species | 1759| 11  | 0.013| 0.083| 0.055| 0.021| Recalculated after Ki et al., 2011 [40] |
| Dinoflagellate   | Peridinium      | 1687| 9   | 0.015| 0.085| NC****| NC****| Ki et al., 2011 [40] |
| Diatom           | Cyclotella      | 1704| 6   | 0.004| 0.034| NC****| NC****| Recalculated after Jung et al., 2010 [41] |
| Diatom           | Discostella     | 1704| 4   | 0.010| 0.015| 0.013| 0.002| Recalculated after Jung et al., 2010 [41] |
| Diatom           | 2 orders*****  | c. 1600| 4 | 0.011| 0.017| NC****| NC****| Jung et al., 2010 [41] |

bp*: Nucleotide bases of the SSU rDNA used for calculations.

n**: Numbers of species (species/phylogenies) used for calculations.

Reference***: Each ref. calculated molecular similarity (we converted values to uncorrected p distance) of individual SSU rDNA.

NC****: Not calculated.

2 orders*****: The values include between-species data set for 2 orders; Thalassiosirales and Naviculales.

doi:10.1371/journal.pone.0060882.t001
areas of Japan (areas B, C, D, E, F and G in Figs. 3 and 4). On the other hand, our 248 strains contained no *G. toxicus*. No *Gambierdiscus* cell was found in samples collected from all stations of the Sea of Japan, except for KN, and some stations along Pacific coast (gray circles in Fig. 3). Details of strains are summarized in Table S5.

The species/phylotype composition plotted onto a map showed a species/phylotype-specific pattern of their distribution (Fig. 4). For example, *G. cf. yasumotoi* and type 3 were restricted in the subtropical area (from the station OM and IR in areas E and G, respectively) and the temperate area (WI in area B), respectively (Fig. 4B). On the other hand, type 1, type 2 and *G. australis* were widespread from the temperate area (areas B, C and D in Fig. 4) to

---

**Figure 3. Map of research area.** Location of each sampling station and its ID as well as presence (yellow circle) or absence (gray circle) of *Gambierdiscus* cells in the sample is shown. Each island is marked as A: Hokkaido, B: Honshu (main Isl.), C: Shikoku, D: Kyushu, E: Okinawa Island, F: Miyako Island, Ikema Island and Irabu Island (from right to left), G: Ishigaki Island and Iriomote Island (from right to left). A: the boreal area, B, C and D: the temperate area, E, F and G: the subtropical area. doi:10.1371/journal.pone.0060882.g003
the subtropical area (areas E, F and G in Fig. 4) in the areas studied (Table 2 and Table S4), with a tendency that type 1 and G. australis were dominant in the subtropical area, whereas type 2 was more abundant in the temperate area (Fig. 4A). Although type 2 exhibited a widespread distribution (Table 2, Fig. 4), they were totally absent at the southern end of our study field (subtropical island areas F and G in Fig. 4). Each sampling station usually held 1–3 species/phylotype (s), with an exceptional case in that four species/phylotypes (type 1, type 2, type 3 and G. australis) were recorded by during samplings on two different dates at a temperate station WI (area B in Fig. 4B).

We compared the occurrence of each species/phylotype with sea water temperature (SWT) measured when the samples were taken. Tendencies that type 1 and G. australis were more abundant in warmer water and that type 2 more commonly occurred in cooler water were found (Table 2), e.g. type 1 cells were isolated from 21.1°C to 32.4°C, G. australis cells were isolated from 19.1°C to 32.4°C, whereas type 2 cells were isolated from 17.2°C to 30.0°C (Table 2). We found no tendency of the occurrence of each species/phylotype with salinity (Table 2).

Toxicity Analyses

The toxicities in mice were tested by means of the dichloromethane soluble fraction (DSF, a fraction expected to contain CTXs) and aqueous methanol soluble fraction (MSF, a fraction expected to contain MTXs) using six strains representing four species/phylotypes, viz., KW070922_1 (Gambierdiscus sp. type 1); M080828_2 and T070411_1 (Gambierdiscus sp. type 2); W19G and W111G (Gambierdiscus sp. type 3); S080911_1 (G. australis). The results of mouse bioassay indicated the presence of toxicity in type 1, type 3 and G. australis but not in type 2 (Table 3 and Table S6). In DSF toxicity, G. australis was the highest (670×10^4 MU 1,000 cells^-1) and type 1 also showed positive result (20×10^4 MU 1,000 cells^-1). In MSF toxicity, both type 1 and G. australis showed positive result (67×10^4 MU 1,000 cells^-1). Although one strain of type 3 (W111G) was positive for MSF toxicity (67 MU 1,000 cells^-1), another strain W19G was negative (Table 3 and Table S6).

Discussion

Genetic Diversity of Japanese Gambierdiscus

A previous phylogeographic study of Japanese Gambierdiscus using the SSU rDNA and the ITS region revealed the existence of two putative allopatric phylotypes: Gambierdiscus sp. type 1 and Gambierdiscus sp. type 2 [22]. On the other hand, we detected five species/phylotypes including the two reported phylotypes of Gambierdiscus (G. australis and G. cf. yasumotoi) and a hitherto unreported phylotype Gambierdiscus sp. type 3 from western and southern coastal areas of Japan. Considering these results, the unexpectedly high genetic diversity of Japanese Gambierdiscus, up to five species/phylotypes in the present study, might simply be explained by the intensiveness of sampling, viz. the more strains established the more probability to encounter something new.

The minimum uncorrected p distances (0.028–0.040) of the SSU rDNA obtained from the pairs between Japanese phylotypes (type 1, type 2 and type 3) and another species, were larger than that of the pair G. yasumotoi vs. G. ruetzleri (0.004) which was the minimum value of between-species of Gambierdiscus. Furthermore, these values were also larger than the minimum p distances calculated for between-species from other protists, e.g. dinoflagellate Peridinium (between 9 species, 0.013) [40], diatom Cyclotella (between 6 species, 0.004) [41], diatom Discostella (between 4 species, 0.010) [41] and 2 orders of diatoms (0.01) [42] (Table 1). Therefore, the minimum p distances obtained from the pairs between Japanese phylotypes and another species are most likely indicative of species level divergence, and Japanese phylotypes will deserve to be described as new species. Currently detailed morphological investigations are ongoing to support the description of these phylotypes (Nishimura et al. unpubl.). It also should be noted that the minimum p distances obtained from the pairs G. yasumotoi vs. G. ruetzleri was the same as or lower than the values obtained from other protists, and was close to those within-species level divergence of Gambierdiscus species.

The mean p distance of the SSU rDNA obtained from between-species (0.139) was larger than those of other protists, e.g. dinoflagellate Peridinium (between 9 species, 0.055) [40], diatom Cyclotella (between 6 species, 0.019) [41], diatom Discostella (between 4 species, 0.013) [41] and 2 orders of diatoms (0.01) [42]. These results suggest that the genetic diversity of Gambierdiscus seems to be larger than other protists.

In relation to Gambierdiscus sp. type 2 from Japanese coastal waters, G. caribaeus GCJJ1 strain from Jeju Island, Korea, Pacific was reported to be closely related with a Japanese strain C-1, which belongs to Gambierdiscus sp. type 2 [22], by Jeong et al. [23]. They also reported that the SSU rDNA sequence of Korean strain GCJJ1, which has not been opened on DNA Data Bank of Japan (DDBJ) yet, was 0.5% different (0.005 for uncorrected p distance in the present study) from that of strain C-1 of Gambierdiscus sp. type 2, whereas the Korean strain GCJJ1 was 2.4–4.0% different (0.024–0.040 for uncorrected p distance) from those of G. caribaeus [23]. On the other hand, morphological observations using SEM suggested that morphology of Korean strain GCJJ1 was similar to those of Belize strains of G. caribaeus. Considering the similar morphology and the molecular divergence between them, the authors suggested that the Korean strain GCJJ1 may be a cryptic species of G. caribaeus. Together with our results that the mean p distance (0.139±0.042) and the minimum p distance (0.004) among Gambierdiscus species, the Korean strain GCJJ1 may belong to Gambierdiscus sp. type 2 rather than G. caribaeus and the strain as well as Japanese strains of type 2 are to be described as a new species.

The SSU rDNA and the LSU rDNA D8–D10 phylogenies indicated that our strains IR4G and Go3 (LSU rDNA D8–D10 sequence was unavailable for the latter strain) belonged to the clade I, along with G. yasumotoi and G. ruetzleri. An empirical study by Litaker et al. [39] proposed that uncorrected p distance between 0.042 and 0.580 substitutions per site in the ITS region are indicative of species level divergence of the ITS region based on their observations among 14 genera of dinoflagellate. Recently Litaker et al. [20] applied the idea with Gambierdiscus, where two morphologically similar but barely and genetically distinguishable species G. yasumotoi and G. ruetzleri had p = 0.07 for the ITS region. As a result of our sequence analyses, we provisionally called our strains IR4G and Go3 (LSU rDNA D8–D10 sequence was unavailable for the latter strain) belonged to the clade I, along with G. yasumotoi and G. ruetzleri. An empirical study by Litaker et al. [39] proposed that uncorrected p distance between 0.042 and 0.580 substitutions per site in the ITS region are indicative of species level divergence of the ITS region based on their observations among 14 genera of dinoflagellate. Recently Litaker et al. [20] applied the idea with Gambierdiscus, where two morphologically similar but barely and genetically distinguishable species G. yasumotoi and G. ruetzleri had p = 0.07 for the ITS region. As a result of our sequence analyses, we provisionally called our strains IR4G and Go3 (LSU rDNA D8–D10 sequence was unavailable for the latter strain) belonged to the clade I, along with G. yasumotoi and G. ruetzleri. An empirical study by Litaker et al. [39] proposed that uncorrected p distance between 0.042 and 0.580 substitutions per site in the ITS region are indicative of species level divergence of the ITS region based on their observations among 14 genera of dinoflagellate. Recently Litaker et al. [20] applied the idea with Gambierdiscus, where two morphologically similar but barely and genetically distinguishable species G. yasumotoi and G. ruetzleri had p = 0.07 for the ITS region.

Distribution of Gambierdiscus in Coastal Areas of Japan

Kuno et al. [22] revealed the existence of two allopatric phylotypes: Gambierdiscus sp. type 1 from the subtropical area
and Gambierdiscus sp. type 2 from the temperate area. On the other hand, we confirmed both of the two phylotypes occur from the subtropical area to the temperate area. In addition to those distributions, we found that G. australis occurs from the subtropical area to the temperate area, whereas, G. cf. yasumotoi and Gambierdiscus sp. type 3 were restricted to the subtropical area and the temperate area, respectively. Additionally, we found a tendency that type 1 and G. australis were dominant in the subtropical area, whereas type 2 was dominant in the temperate area. Considering those trends of the distribution of each species/phylotype in Japan, different growth response of each species/phylotype to various environmental parameters, such as SWT and salinity so on, would determine those trends of the distribution. Comparing those distributions with SWT when each cell was isolated, type 1 and G. australis cells were isolated from warmer waters, while type 2 cells were isolated from cooler waters. Additionally, type 2 cells were isolated from KN (35°41’31 N, 135°18’16 E), in which Hatayama et al. [38] has also observed Gambierdiscus cells. The sampling station is the most northerly location in the world where Gambierdiscus spp. have been reported so far. These results indicate that type 2 appears to prefer cooler waters to warmer waters because of their growth response which probably plays a crucial role in their predomination around the temperate areas in Japan. To confirm this hypothesis, comparative culture experiments with type 1, type 2, type 3 and G. australis are currently ongoing (Yoshimatsu et al. unpubl.). Additionally, we found no Gambierdiscus cell from Japanese northern stations (HO, HF, IW, NS and NC), where the number of samples was poorly represented in our collection. The absence of Gambierdiscus from our northern samples could also be explained by their restricted distribution in warm waters; so far there is no Gambierdiscus found from boreal area.

Toxicity

Chinain et al. [18] applied mouse bioassay (MBA) for detection of toxicities of four Gambierdiscus species (G. toxicus, G. pacificus, G. polynesiensis and G. australis) isolated from French Polynesia, Pacific and revealed that G. pacificus, G. polynesiensis and G. australis showed both toxicities of DSF (a fraction expected to contain CTXs) and MSF (a fraction expected to contain MTSs), whereas G. toxicus showed only MSF toxicity. In the present study, MBA results of Japanese Gambierdiscus revealed that three species/phylotypes had toxicities (G. australis, Gambierdiscus sp. type 1 and Gambierdiscus sp. type 3) and a phylotype (Gambierdiscus sp. type 2) was thought to be non-toxic. Unfortunately, G. cf. yasumotoi could not be assessed for its toxicity, because mass culturing was not successful. However, a strain of G. yasumotoi isolated from Singapore was found to be potentially toxic [17].

In DSF toxicities among Japanese Gambierdiscus, the toxicity of G. australis was stronger than that of type 1. Additionally, for type 3 which was revealed to be “non-toxic” for DSF in the present study, a strain W9G exhibited progressive paralysis of mice or death of mice however the proportion that died did not exceed 50%. The DSF toxicity of Japanese G. australis was approx. 170-fold stronger than that of G. australis isolated from French Polynesia [18]. This suggests that the toxicity of a Gambierdiscus species is possibly variable being related with location where each strain was isolated. Furthermore, the degree of DSF toxicity of Japanese G. australis followed the toxicities of G. polynesiensis strains, which are known as ‘super-toxic strains [43]’. The DSF toxicity of type 1 followed that of Japanese G. australis. In MSF toxicities among Japanese Gambierdiscus, the toxicities of G. australis, type 1 and type 3 were the same value, furthermore those toxicities were stronger than those of G. toxicus, G. pacificus, G. polynesiensis and G. australis isolated from French Polynesia [18]. Considering these results, it was revealed that species/phylotypes which have relatively strong DSF and MSF toxicities so far may distribute around Japanese coastal areas.

Conclusion/Future Prospects

As a result of phylogenies of the SSU rDNA and the LSU rDNA D8–D10, we revealed that five species/phylotypes including two reported phylotypes (Gambierdiscus sp. type 1 and Gambierdiscus sp. type 2 [22]), two species of Gambierdiscus (G. australis and G. cf. yasumotoi).
Table 3. Toxicities of Japanese *Gambierdiscus* species/phylotypes tested by a mouse bioassay. One MU is defined as the LD$_{50}$ dose for a 20-g mouse over 24 h.

| Species/phylotype | Strain   | DSF toxicity* ($\times 10^{-4}$ MU/1,000 cells) | MSF toxicity** ($\times 10^{-4}$ MU/1,000 cells) | Reference          |
|-------------------|----------|-----------------------------------------------|------------------------------------------------|-------------------|
| *Gambierdiscus* sp. type 1 | KW070922_1 | 20                                            | 67                                              | This study        |
| *Gambierdiscus* sp. type 2 | M080828_2  | ***                                          | ***                                             | This study        |
|                     | T070411_1 | ***                                          | ND***                                           | This study        |
| *Gambierdiscus* sp. type 3 | W19G   | ***                                          | ***                                             | This study        |
|                     | W111G   | ***                                          | 67                                              | This study        |
| *Gambierdiscus australis* | S080911_1 | 670                                          | 67                                              | This study        |
|                    | RAV-92  | 4                                            | 0.2                                             | Chinain et al., 1999 [18] |
| *Gambierdiscus toxicus* | GTT-91  | ***                                          | 0.7                                             | Chinain et al., 1999 [18] |
|                    | REN-1   | ***                                          | 1.7                                             | Chinain et al., 1999 [18] |
|                     | TUR     | ***                                          | 0.6                                             | Chinain et al., 1999 [18] |
| *Gambierdiscus pacificus* | HO-91   | 9                                            | 0.7                                             | Chinain et al., 1999 [18] |
| *Gambierdiscus polynesiensis* | TB-92  | 1500                                         | 0.1                                             | Chinain et al., 1999 [18] |
|                     | RG-92   | 800                                          | 0.06                                            | Chinain et al., 1999 [18] |

DSF*: Dichloromethane soluble fraction (DSF) toxicities correspond to CTXs toxicity. MSF**: Aqueous methanol soluble fractions (MSF) toxicities correspond to MTXs toxicity. ***: Not detected. ND****: Not done.

doi:10.1371/journal.pone.0060882.t003

as *yasumotoi* and a hitherto unreported phylotype *Gambierdiscus* sp. type 3 distribute around Japanese coastal areas, especially western and southern areas. Out of Japanese five species/phylotypes, the three phylotypes (type 1, type 2 and type 3) that showed species level genetic divergence by calculating uncorrected genetic distance using these genes will deserve to be described as new species. Also the distribution of Japanese *Gambierdiscus* was revealed; *G. cf. yasumotoi* and type 3 distributed allopatrically to the subtropical area and the temperate area, respectively. On the other hand, type 1, type 2 and *G. australis* occurred from the subtropical area to the temperate area, with a tendency that type 1 and *G. australis* were dominant in the subtropical area, whereas type 2 was dominant in the temperate area. We revealed a surprising diversity of Japanese *Gambierdiscus*, and the distribution of five species/phylotypes displayed clear geographical patterns in the subtropical and temperate areas. For revealing toxicities of those species/phylotypes, MBA was conducted and revealed that *G. australis* and type 1 had toxicities of DSF and MSF, and type 3 had only toxicity of MSF, whereas no toxicities of both fractions were detected from type 2. In the DSF toxicities, *G. australis* was stronger than that of type 1. So far, occurrences of CFP incidents have been mainly reported from the subtropical area of Japan that is suggested that one of agents of CFP incidents occurred in *G. australis*. Comparative LC-MS studies on extracts from both cultured *Gambierdiscus* and ciguateric fish are required to elucidate as to whether the occurrence of CFP is linked to the toxins produced by *Gambierdiscus*.

As a whole, the SSU rDNA and the LSU rDNA D8–D10 as genetic marker is recommended for further use, i.e. species/phylotype-level comparison such as molecular systematic/taxonomy within the genus. Now we are trying to establish the molecular-based monitoring system using quantitative polymerase chain reaction (qPCR) assay for the detection of each toxic species/phylotype of *Gambierdiscus* in Japanese coastal areas.

**Materials and Methods**

**Ethics Statement**

No specific permits were required for the described field studies. No specific permission was required for any locations and activity. The locations are not privately owned or protected in any way. No activity during field study involved any endangered species or protected species. The Animal Use Protocol (AUP) for handling mice was approved by the Animal Ethics Committee (approval ID: D-00068) of Kochi University.

**Isolation and Establishment of *Gambierdiscus* Strains**

Macroalgal substrata were collected mainly from the southern part of Japan between 2006 and 2011. Details of the sampling stations are shown in Table S4. In the laboratory the macroalgae were placed in a plastic bottle and vigorously shaken to cause epiphytes, including *Gambierdiscus*, to detach from the substrata. The resultant suspension was sieved twice, firstly through 150 µm and then through 20 µm Nitex meshes. Materials retained on the second mesh filter were resuspended in filtered seawater. *Gambierdiscus* cells placed in a well/flat bottom microplate (Asahi Glass, Tokyo, Japan) were isolated under an inverted microscope referring to the morphological criteria of the genus *Gambierdiscus* described by Adachi and Fukuyo [14]. All the clonal strains were maintained with Daigo IMK (Nihon Pharmaceutical, Tokyo, Japan), IMK/2, IMK/4 or soil-extract-added IMK/4 medium using GF/F-filtered sea water (salinity of 31 ± 1 in polypropylene (PP)-capped test tubes (25 × 150 mm) with a flat bottom (Marineu, Osaka, Japan) containing 20 ml medium at 25°C, with 100 µmol photons m⁻² s⁻¹ from cool-white tubes; the photoperiod was 12:12 h L:D.
DNA Extraction, PCR, Cloning and Sequence

To amplify the SSU rDNA and the LSU rDNA D8–D10 we performed direct PCR, which used intact cells for PCR template instead of genomic DNA allowing us to skip the DNA extraction process, in order to analyze large numbers of isolates quicker and more efficiently. For harvesting cells for template, 1 ml of medium containing Gambierdiscus cells was centrifuged to make a pellet, which was subsequently washed twice with sterile water. A small fraction was picked from the pellet by a micropipette and transferred into a PCR tube that contained a 25-μl mixture as below. In case direct PCR failed to amplify the SSU rDNA and/or the LSU rDNA D8–D10, and the ITS region for IR4G, genomic DNA was extracted from the strains using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

The SSU rDNA was amplified by using 50 μM of oligonucleotide primers Dino5’UF [44] and 18 ScomR1 [45] (Table 4). PCR reactions typically contained a 25-μl mixture: 0.5 μl of MightyAmp DNA Polymerase (1.25 U/μl, Takara Bio, Shiga, Japan); primers as above (7.5 pmol each); 12.5 μl of 2×MightyAmp buffer (Mg²⁺, dNTP mix) which contains magnesium chloride (4 mM) and dNTPs (800 μM each). The PCR cycling comprised of an initial 2 min heating step at 98°C, followed by 35 cycles: 98°C for 10 sec, 68°C for 110 sec, and a final extension at 68°C for 5 min. The PCR amplification of the SSU rDNA for strains from several sampling stations (MME, OBS, OM, OD, MYA and MYI, Table S4) was performed at University of the Ryukyus using primers and methods described in Takano and Horiguchi [46]. The LSU rDNA was amplified by using 50 μM of oligonucleotide primers FD8 and RB [18] (Table 4). The PCR cycling comprised of an initial 2 min heating step at 98°C, followed by 25 cycles: 98°C for 10 sec, 55°C for 15 sec and 68°C for 40 sec, and a final extension at 68°C for 5 min. The ITS region was amplified by using 50 μM of oligonucleotide primers ITSA [47] and D2C [48] (Table 4). The PCR cycling comprised of an initial 2 min heating step at 98°C, followed by 35 cycles: 98°C for 10 sec, 55°C for 15 sec and 68°C for 75 sec, and a final extension at 68°C for 5 min.

The quantity and length of products were examined by agarose gel electrophoresis against known standards. Excess primers and dNTPs were removed from PCR product using High Pure PCR Cleanup Micro Kit (Roche, Tokyo, Japan). Clones were screened for inserts by PCR amplification with plasmid primers U19 and pUCM13R (Table 4).

BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Japan, Chiba, Japan) was used for the sequence of the PCR products and the clones. Primers and excess dye-labeled nucleotides were removed using the Performa DTR V3 clean-up system (Edge Biosystems, Gaithersburg, MD).

For easy and quick phylotyping of strains, approximately 500 bp including the most variable 300 bp region of the SSU rDNA [44] were sequenced by internal sequencing primer G10R [49] (Table 4). Sequencing products were run on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems Japan, Chiba, Japan). Forward and reverse reads were edited and aligned using SeqMan (DNASTAR, Madison, WI). All the information of clonal strains, including locality, water temperature and source sample are listed in Table S5.

Phylogeny and Sequence Analyses

The SSU rDNA and the LSU rDNA D8–D10 sequences were aligned using ClustalW [50] with publicly available ones retrieved from DNA Data Bank of Japan (DDBJ). In both rDNA datasets, the 5’ and 3’ ends were manually aligned to truncate and refine the both ends. Finally, ambiguously aligned positions were excluded, resulting in 190 taxa/1757 nucleotides (32 bp–1762 bp site of *Ftusteria piscicida*, AY245693) followed the alignment used in Litaker et al. [44] and 136 taxa/946 nucleotides (1980 bp–2978 bp site of *Prorocentrum micans*, X16106) followed the alignment used in Lenaers et al. [51] in the SSU rDNA and the LSU rDNA D8–D10 dataset, respectively.

MrBayes 3.1.2 [52,53] was used for Bayesian inference (BI) to estimate the posterior probability distribution using Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) [53]. MCMCMC from a random starting tree were used in this analysis with two independent runs and 1 cold and 3 heated chain with temperature set 0.2. Trees were sampled every 100 th generation. To increase the probability of chain convergence, we sampled at least 10,000 trees after the standard deviation values of the two runs dipped below 0.01 to calculate the posterior probabilities. RAxML-VI-HPC, v7.0.4 [54] was used for Maximum likelihood (ML) analyses. We conducted a rapid Bootstrap analysis and search for the best-scoring ML tree in one single run with -f a option for 100 repeats. MrModeltest 2 [55] was used to determine the most appropriate model of sequence evolution. The best-fit model according to the Akaike Information Criterion (AIC) was GTR+G, and this was used for BI and ML analyses.

With the ITS region we calculated the uncorrected genetic distance (p) to consider substitutions, gaps and ambiguous bases for calculations among G. yasumotoi, G. ruetzleri and Japanese strain IR4G, in order to find the closest relative of these strains. The ITS region sequences of G. yasumotoi and G. ruetzleri were obtained from Fig. 74 in Litaker et al. [20]. A consensus of the ITS region sequence for six separate clones (sequences obtained via cloning) of IR4G was used for the analysis to reduce any bias due to the inclusion of pseudogene sequences. Additionally we also calculated p distance using uncorrected genetic distance (UGD) model (p-distance model in MEGA 5.05 [56]) among Gambierdiscus species in that strict consensus sequences were obtained both for each species using the SSU rDNA and the LSU rDNA D8–D10 and revealed the p distances of within-species and between-species. Then, we calculated the p distances between each Japanese phylotype and another species. All positions containing alignment gaps and missing data were eliminated only in pairwise comparisons (Pairwise deletion option in MEGA 5.05 [56]). For the UGD model of the SSU rDNA and the LSU rDNA D8–D10 calculation in the present study, using MEGA 5.05, gaps and ambiguous bases were not considered.

Toxicity Analyses

Six strains representing four species/phylotypes, viz., KW070922_1 (Gambierdiscus sp. type 1); M080828_2 and T070411_1 (Gambierdiscus sp. type 2); W19G and W111G (Gambierdiscus sp. type 3); S089911_1 (G. austriate), were maintained in glass Petri dishes (SANSYO, Tokyo, Japan) filled with 20 mL of Daigo IMK medium under culturing conditions described as above. Cells in the late stationary phase (approx. 30 days) were harvested by filtration using 20 μm Nitex mesh and/or centrifuging. Toxic extracts were prepared by extraction from cell pellets, by using the method modified after Chinai [18], thrice in methanol (2 mL for a total biomass of 1.0 × 10⁶ cells) and thrice in aqueous methanol (MeOH:H₂O 9:1) (1 mL for a total biomass of 1.0 × 10⁶ cells) under sonication, for 15 min. each. After dividing toxic extracts into the required number of specimens, the extract was evaporated, a solvent portion was applied to the resulting residue using 0.4 mL of dichloromethane (CH₂Cl₂) once and 0.2 mL of aqueous methanol (MeOH:H₂O 6:4) twice. During liquid-liquid partitions, the dichloromethane and aqueous methanol phases, in which CTXs and MTXs are recovered respec-
tively, were handled with extreme care in order to limit carry over of MTXs into the dichloromethane phase, and vice versa. The dichloromethane soluble fraction (DSF) and aqueous methanol soluble fraction (MSF) were evaporated, respectively. The DSF and MSF of six strains of *Gambierdiscus* sp. were tested for their toxicity using mouse bioassay. Three or five mice (male, ddY, 20 g; Japan SLC, Inc, Shizuoka, Japan) were intraperitoneally administered with a single dose of toxic extract dissolved in 500 µl of a 0.85% saline solution containing 1% Tween 60. Mice were observed over 24 h and signs and time of death recorded. Fractions were considered non-toxic if injection of a maximal dose was not lethal. Total lethality was expressed in mouse units (MU). One MU is defined as the i.p. LD<sub>50</sub> dose for a 20 g mouse over 24 h.

**Table 4. Oligonucleotide primers used for PCR amplification and DNA sequencing.**

| Primer name | Synthesis direction | Sequence (5’–3’) | Anneals to | Reference |
|-------------|---------------------|------------------|------------|-----------|
| **The ITS1-5.8S-ITS2 rDNA** | | | | |
| ITS A | Forward | GTA ACA AGG THT CCG TAG GT | 22–41* | Sato et al, 2011 [47] |
| type4ITSICF | Forward for sequencing | TTG TTG GTT CCT CCA GA | 83–102* | This study |
| inner5.8S_F | Forward for sequencing | AAA TTG CAG AAT YCC GTG AG | 306–325* | This study |
| inner5.8S_R2 | Reverse for sequencing | TGA TCT AGC GAA TTC T GCC | 311–328* | This study |
| type4ITSICR | Reverse for sequencing | GTC TGC CAG TGT CAC AAT GC | 473–492* | This study |
| ITS B | Reverse for sequencing | AKA TGC TTA ART TCA GCR GG | 542–561* | Sato et al, 2011 [47] |
| D2C | Reverse | CCT TGG TCC GTG TTT CAA GA | 714–733** | Scholin et al, 1994 [48] |
| **The SSU rDNA** | | | | |
| Dino5′UF | Forward | CAA CCT GGT GAT CCT GCC AGT | 1–23*** | Litaker et al, 2005 [49] |
| 18ScomF1 | Forward for sequencing | GCT TGT CTC AAA GAT TAA GCC TAG C | 32–56*** | Zhang et al, 2005 [45] |
| 18S_G53F | Forward for sequencing | TGC ATG TCT CAG CTT AAG TG | 54–73*** | This study |
| G10F | Forward for sequencing | TGG AGG GCA AGT CTG GTC | 549–566*** | This study |
| 18S_G623F | Forward for sequencing | GTC AAG AGC CTA TGA GTT GGA | 618–638*** | This study |
| G17F | Forward for sequencing | ATA CCG TCM TAG TCT TAA CC | 1007–1026*** | Modified after Litaker et al, 2003 [44] |
| 18S_G1249F | Forward for sequencing | GGA TTG ACA GAT TGA CAG CT | 1232–1251*** | This study |
| G18F | Forward for sequencing | CAA TAA CAG GTC TGT GAT GC | 1426–1445*** | Litaker et al, 2003 [44] |
| 18S_G371R | Reverse for sequencing | ACC CTC ATC CTC CTT CAC CT | 359–378*** | This study |
| G10R | Reverse for sequencing | CCG CGG CTG CTG CCA CCA GAC | 559–579*** | Litaker et al, 2005 [49] |
| 18S_G781R | Reverse for sequencing | AAA CAC CTG CTT TGA ACA C | 768–786*** | This study |
| G17R | Reverse for sequencing | GTT TAT GGT TAA GAC TAK GAC GG | 1010–1032*** | This study |
| 18S_G1301R | Reverse for sequencing | CAC TCC ACC AAC TAA GAA CG | 1279–1303*** | This study |
| G18R | Reverse for sequencing | GCA TCA CAG ACC TGT TAT TG | 1426–1445*** | Litaker et al, 2005 [49] |
| 18S_G1781R | Reverse for sequencing | GAA ACC TTG TTA CGA CTT CT | 1758–1777*** | This study |
| 18ScomR1 | Reverse | CAC CTG AAA CTT TGT TAC GAC | 1762–1785*** | Zhang et al, 2005 [45] |
| **The D8–D10 region of the LSU rDNA** | | | | |
| FDB | Forward | GGA TTG GCT CTG AGG GTT GGG | 1980–2000** | Chinain et al, 1999 [18] |
| GLD8_421F | Forward for sequencing | ACA GCC AAG GGA ACG GGC TT | 2395–2414** | This study |
| GLD8_677R | Reverse for sequencing | TGG GCC GCC CCA GCC AAA CTG | 2645–2664** | This study |
| RB | Reverse | GAT AGG AAG AGC CGA CAT CGA | 2905–2925** | Chinain et al, 1999 [18] |
| **T-Vector pMD20** | | | | |
| U19 | Forward | GTT TTT CCC AGT CAC GAC G | In the vector | Applied Biosystems |
| pUCM13R | Reverse | CAG GAA ACA GCT ATG AC | In the vector | Promega |

*: Annealing site in the ITS1-5.8S-ITS2 rDNA sequence of *Gambierdiscus yasumotoi* GYASU, GU968498 (Vandersea et al, 2010 Direct Submission).

**: Annealing site in the LSU rDNA sequence of *Prorocentrum micans*, X16108 [Lenaers et al, 1989 [51]].

***: Annealing site in the SSU rDNA sequence of *Pfiesteria piscicida*, AY245693 (Litaker et al, 2003 [44]).

doi:10.1371/journal.pone.0060882.t004

**Supporting Information**

**Figure S1** Maximum likelihood (ML) phylogeny of the D8–D10 region of the LSU rDNA of *Gambierdiscus species/phylotypes*. Nodal supports are of ML analysis. Nodes with strong supports (pp/bt = 1.00/100) are shown as thick lines. For sequences obtained via cloning, a variant ID, starting with C, is shown followed by strain ID (i.e. T080908_1_C1). Sequences obtained in the present study are indicated in color. (TIF)

**Table S1** Estimation of genetic distances of the SSU rDNA over the p distance calculated by uncorrected genetic distance (UGD) model among and within 69
consensus sequences (=69 strains; each consensus sequence was constructed with cloned sequences and/or directly sequence of each strain) of Gambierdiscus species/phylotypes.

Table S2 Summary of genetic distances of the D8–D10 region of the LSU rDNA over the p distance calculated by uncorrected genetic distance (UGD) model among and within 51 consensus sequences (=51 strains; each consensus sequence was constructed with cloned sequences and/or directly sequence of each strain) of Gambierdiscus species/phylotypes.

Table S3 Estimation of genetic distances of the D8–D10 region of the LSU rDNA over the p distance calculated by uncorrected genetic distance (UGD) model among and within 61 consensus sequences (=61 strains; each consensus sequence was constructed with cloned sequences and/or directly sequence of each strain) of Gambierdiscus species/phylotypes.

Table S4 Details of macroalgal sample collection.

References

1. Ragelis EP (1984) Ciguatera seafood poisoning: overview. In: Ragelis EP, eds. Seafood Toxins, American Chemical Society, Washington D.C. 22–36.
2. Bagus R (1993) Ciguatera fish poisoning. In: Falkner IR, eds. Algal Toxins in Seafood and Drinking Water, Academic Press, New York. 105–115.
3. Yasumoto T, Satake M (1996) Chemistry, etiology and determination methods of ciguatera toxins. J Toxicol - Tox Rev 15: 91–107.
4. Fleming LE, Baden DG, Bean JA, Weisman R, Blythe DG (1998) Seafood toxin resources. In: Inter-governmental Oceanographic Commission. Commission of UNESCO. 243–248.
5. Lehane L, Lewis RJ (2000) Ciguatera: recent advances but the risk remains. Int J Food Microbiol 61: 91–125.
6. Chinnai M, Darius HT, Ung A, Fouc MT, Revel T, et al. (2010) Ciguatera risk management in French Polynesia: The case study of Rarotonga Island (Australas Archipelago). Toxicon 56: 674–690.
7. Tester PA, Feldman RL, Nau AW, Kibler SR, Litaker RW (2010) Ciguatera fish poisoning and sea surface temperatures in the Caribbean Sea and the West Indies. Toxicon 56: 698–710.
8. Litaker RW (2001) The changing face of ciguatera. Toxicon 39: 97–106.
9. Oshiro N, Nakanoo K, Sasaki T, Tamanaka K, et al. (2010) Ciguatera incidence and fish toxicity in Okinawa, Japan. Toxicon 56: 656–661.
10. Tanizawa S (2008) The occurrence of palytoxin-like poisoning and ciguatera in parts of the main land of Japan. Nippon Suisan Gakkaishi 74: 917–919. (In Japanese).
11. Ishikawa A, Kurashima A (2010) Occurrence of the toxic benthic dinoflagellate Gambierdiscus toxicus in Ayo Bay, central part of Japan. Bull Jpn Soc Fish Oceanogr 74: 13–19. (In Japanese).
12. Oshiro N, Matsuo T, Sakugawa S, Yogi K, Matsuda S, et al. (2011) Ciguatera fish poisoning on Kakeroma Island, Kagoshima Prefecture, Japan. Trop Med Health 39: 53–57. (In Japanese).
13. Yasumoto T, Nakajima, Bagus R, Adachi R (1979) The thecal structure of a marine toxic dinoflagellate Gambierdiscus yasumotoi gen. et sp. nov. collected in a ciguatera-endemic area. Bull Jpn Soc Fish 59: 637–650.
14. Adachi R, Fukuyo Y (1979) The thecal structure of a marine toxic dinoflagellate Gambierdiscus yasumotoi gen. et sp. nov. collected in a ciguatera-endemic area. Bull Jpn Soc Fish 59: 637–650.
15. Fraga S, Rodrı´guez F, Caillaud A, Dioge`ne J, Raho N, et al. (2011) Gambierdiscus spp. clonal strains. (XLS)
16. Holmes MJ (1999) Gambierdiscus yasumotoi sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from southeastern Asia. J Phycol 34: 661–668.
17. Chinnai M, Faust MA, Paulus S (1999) Morphology and molecular analyses of three toxic species of Gambierdiscus (Dinophyceae): G. pacificus, sp. nov., G. antilus, sp. nov., and G. puhinuru, sp. nov. J Phycol 35: 1282–1296.
18. Chinnai M, Faust MA, Paulus S (1999) Morphology and molecular analyses of three toxic species of Gambierdiscus (Dinophyceae): G. pacificus, sp. nov., G. antilus, sp. nov., and G. puhinuru, sp. nov. J Phycol 35: 1282–1296.
19. Chinnai M, Faust MA, Paulus S (1999) Morphology and molecular analyses of three toxic species of Gambierdiscus (Dinophyceae): G. pacificus, sp. nov., G. antilus, sp. nov., and G. puhinuru, sp. nov. J Phycol 35: 1282–1296.
20. Litaker RW, Vanderswa MA, Faust MA, Kibler SR, Chinnai M, et al. (2009) Toxicity of Gambierdiscus including four new species, Gambierdiscus carabinus, Gambierdiscus carinatus, Gambierdiscus caribilis, and Gambierdiscus ruetzleri (Gonyaulacales, Dinophyceae). Phylozyma 48: 344–390.
21. Litaker RW, Vanderswa MA, Faust MA, Kibler SR, Nau AW, et al. (2010) Global distribution of ciguatera causing dinoflagellates in the genus Gambierdiscus. Toxicon 56: 711–730.
22. Kuno S, Kamikawa R, Yoshimatsu S, Sagara T, Nishio S, et al. (2010) Genetic diversity of Gambierdiscus spp. (Gonyaulacales, Dinophyceae) in Japanese coastal areas. Physiol Res 58: 44–52.
23. Jeong HJ, Lim AS, Jang SH, Yih WH, Kang SS, et al. (2012) First report of the epiphytic dinoflagellate Gambierdiscus carbinus in the temperate waters off Jeju Island, Korea. Morphology and molecular characterization. J Eukaryot Microbiol 59: 657–663.
24. Parson ML, Aligazaki K, Bottein MYD, Fraga S, Morton SL, et al. (2012) Gambierdiscus and Ostreopsis: Reassessment of the state of knowledge of their taxonomy, geophysics, ecosphysiology, and toxicology. Harmful Alg 14: 107–129.
25. Naik RK, Hegde S, Anil AC (2011) Dinoflagellate community structure from the stratified environment of the Bay of Bengal, with special emphasis on harmful algal bloom species. Environ Monit Assess 183: 125–130.
26. Roeder K, Erler, Kibler S, Tester P, The HV, et al. (2010) Characteristic profiles of Ciguatera toxins in different strains of Gambierdiscus spp. Toxicon 56: 731–738.
27. Caillaud A, Yasumoto T, Diogène J (2010) Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in Gambierdiscus spp. Toxicon 56: 36–44.
28. Poisidio GN, Dimmao LM (2004) Population densities of potentially toxic epiphytic dinoflagellates in Lingsat Reef, La Union Province, Philippines. Philipp Agric Sci 87: 149–159.
29. Uus G, Leaw CP, Aamit A (2002) Increasing importance of harmful algal blooms in Malaysia. In: Proceedings of the Regional Symposium on Environment and Natural Resources 10–11th April 2002 Malaysia. 144–153.
30. Mohammad-Noor N, Daugbjerg N, Moestrup Ø, Anton A (2004) Marine bloom episodes of dinoflagellates from Malaysia - a study of live cultures and preserved samples based on light and scanning electron microscopy. Nord J Bot 24: 629–634.
31. Leaw CP, Lim PT, Tan TH, Tuan-Halim TN, Cheng KW, et al. (2011) First report of the benthic dinoflagellate Gambierdiscus belizeanus (Gonyaulacales: Dinophyceae) for the east coast of Sabah, Malaysian Borneo. Physiol Res 59: 143–146.
32. Kim HS, Yih W, Kim JH, Myung G, Jeong HJ (2011) Abundance of epiphytic dinoflagellates on coral reefs and macroalgal samples. Toxicon 56: 711–730.
34. Hara Y, Horiguchi T (1982) A floristic study of the marine microalgae along the coast of the Izu Peninsula. Mem Natl Sci Mus 15: 99–108. (In Japanese).
35. Koike K, Ishimaru T, Murano M (1991) Distributions of benthic dinoflagellates in Akajima Island, Okinawa, Japan. Bull Japan Soc Sci Fish 57: 2261–2264.
36. Fukuyo Y, Imai I, Kodama M, Tamai K (2002) Red tides and other harmful algal blooms in Japan. In: Taylor FJR, Trainer VL, eds. Harmful algal blooms in the PICES region of the North Pacific. PICES Scientific Report No. 23. North Pacific Marine Sci Org (PICES), Canada. 7–28.
37. Ohnara T, Nakahama Y, Fukuyo Y (2010) Ciguatera causative species found in main land of Japan. In: GEOHAB Meeting Honolulu, Hawaii, USA, 21–23 June 2010. Available: http://www.scor-int.org/GEOHAB_BHAV_Program_Book.pdf. Accessed 13 November 2012.
38. Hatayama Y, Ishikawa A, Natsuike M, Takeichi Y, Ajisaka T, et al. (2011) First report of the benthic dinoflagellate of the genus Gambierdiscus from western Wakasa Bay in the Sea of Japan. Nippon Suisan Gakkaishi 77: 685–687. (In Japanese).
39. Litaker RW, Vandersea MW, Kibler SR, Reece KS, Stokes NA, et al. (2007) Recognizing dinoflagellate species using ITS rDNA sequences. J Phycol 43: 344–355.
40. Ki JS, Park MH, Han MS (2011) Discriminative power of nuclear rDNA sequences for the DNA taxonomy of the dinoflagellate genus Peridinium (Dinophyceae). J Phycol 47: 426–435.
41. Jung SW, Han MS, Ki JS (2010) Molecular genetic divergence of the centric diatom Cyclotella and Diatoma (Bacillariophyceae) revealed by nuclear ribosomal DNA comparisons. J Appl Phycol 22: 319–329.
42. Moniz MBJ, Kaczmarska I (2009) Barcoding diatoms: Is there a good marker? Mol Ecol Resour 9: 65–74.
43. Chijin A, Darius HT, Ung A, Crucchet P, Wang Z, et al. (2010) Growth and toxin production in the ciguatera-causing dinoflagellate Gambierdiscus polynesiensis (Dinophyceae) in culture. Toxicon 56: 739–750.
44. Litaker RW, Vandersea MW, Kibler SR, Reece KS, Stokes NA, et al. (2003) Identification of Pfiesteria piscicida (Dinophyceae) and Pfiesteria-like organisms using internal transcribed spacer-specific PCR assays. J Phycol 39: 754–761.
45. Zhang H, Bhattacharya D, Lin S (2005) Phylogeny of dinoflagellates based on mitochondrial cytochrome b and nuclear small subunit rDNA sequence comparisons. J Phycol 41: 411–420.
46. Takano Y, Horiguchi T (2003) Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. J Phycol 42: 251–256.
47. Sato S, Nishimura T, Uehara K, Sakakari H, Tawong W, et al. (2011) Phylogeography of Ostracopus along west Pacific coast, with special reference to a novel clade from Japan. PLoS ONE 6: e27983.
48. Scholin CA, Herzog M, Sogin M, Anderson DM (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. J Phycol 30: 999–1011.
49. Litaker RW, Steidinger KA, Mason PL, Landberg JH, Shields JD, et al. (2005) The reclassification of Pfiesteria shumwayae (Dinophyceae): Pseudopfiesteria, gen. nov. J Phycol 41: 643–651.
50. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.
51. Lenaers G, Maroteaux I, Michot B, Herzog M (1989) Dinoflagellates in evolution. A molecular phylogenetic analysis of large subunit ribosomal RNA. J Mol Evol 29: 40–51.
52. Hulsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.
53. Ronquist F, Hulsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
54. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.
55. Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
56. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.