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Morphology of *Gambierdiscus excentricus* (Dinophyceae) with emphasis on sulcal plates

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Abstract: *Gambierdiscus excentricus* is an epibenthic dinoflagellate able to produce ciguatoxin and maitotoxin-like compounds that are responsible for ciguatera fish poisoning. Morphological descriptions and molecular characterization of two *G. excentricus* strains isolated from Brazil and maintained in culture were provided. The most complete description of the morphology of the sulcal region of *Gambierdiscus* based on light and scanning electron microscopy was presented. The sulcal area morphology and nomenclature used by different authors to name the sulcal plates in *Gambierdiscus* were reviewed. Two small sulcal plates (S.m.a. and S.m.p.) were shown for the first time. Phylogenetic trees based on D1–D3 and D8–D10 large subunits of ribosomal RNA gene sequences showed that the strains of *G. excentricus* from Brazil clustered with strains of *G. excentricus* isolated from its type locality, the Canary Islands. Both phylogenetic trees reconstructed the same relationships among all the formally described *Gambierdiscus* species and *Gambierdiscus* sp. ribotype 2 and *Gambierdiscus* sp. type 2.

Key Words: Ciguatera, Epibenthic dinoflagellates, LSU, Sulcal morphology

INTRODUCTION

Gambierdiscus Adachi & Fukuyo is a genus of epibenthic dinoflagellate associated with substrata that include macroalgae, sand, debris and coral reefs. It is usually found with other toxigenic dinoflagellates including *Ostreopsis* Schmidt, *Coolia* Meunier, *Prorocentrum* Ehrenberg and *Amphidinium* Claperède & Lachmann (Fraga *et al*. 2011). The genus was considered restricted to tropical and subtropical environments but it was recently detected in the Mediterranean Sea (Aligizaki & Nikolaidis 2008), the Canary Islands (Fraga *et al*. 2011; Fraga & Rodríguez 2014) and in temperate areas of Japan (Nishimura *et al*. 2013).

Gambierdiscus species produce ciguatoxins (Murata *et al*. 1989) that have been generally regarded as the primary toxins responsible for ciguatera fish poisoning (CFP). CFP occurs after consumption of herbivorous and carnivorous fish that have bioaccumulated ciguatoxins through the food web. As these toxins bioaccumulate, they are frequently modified to form several major, and numerous minor, chemical congeners whose toxicity can vary significantly (Lehane & Lewis 2000). CFP is characterized by diverse symptoms that include gastrointestinal, cardiovascular and neurological effects (Lehane & Lewis 2000). It is widespread in tropical and subtropical marine areas, affecting mainly the Caribbean Sea, Pacific Ocean and Indian Ocean (Lewis 2006). It is estimated that from 50,000 to 500,000 people are affected by CFP every year, and it is the most frequently reported nonbacterial illness associated with seafood consumption worldwide (Fleming *et al*. 1998). Moreover, a 60% increase in CFP in Pacific Island nations has been reported over the last decade (Skinner *et al*. 2011), and the predicted positive response of *Gambierdiscus* to global climate change (Tester *et al*. 2010) may potentially increase their distribution and the risk of ciguatera.

Gambierdiscus was considered a monospecific genus for many years, with *Gambierdiscus toxicus* Adachi & Fukuyo as the only described species (Adachi & Fukuyo 1979). The taxonomic state of *Gambierdiscus* was reviewed by Litaker *et al*. (2009), who presented distinguishing morphological features among species and provided a synthesis of morphological and genetic differences. The genus included 13 described species (Litaker *et al*. 2009; Fraga *et al*. 2011; Fraga & Rodríguez 2014; Nishimura *et al*. 2014) plus seven genotypes (Kuno *et al*. 2010; Litaker *et al*. 2010; Nishimura *et al*. 2013; Xu *et al*. 2014). Based on morphology, the genus included two globular and 11 anterio-posteriorly compressed (discoid) species. Recently, Gómez *et al*. (2015) reported a third globular species and proposed the transfer of the globular species to the new genus *Fukuyoa* F. Gómez, D. Qiu, R.M. Lopes & S. Lin. For identification purposes, *Gambierdiscus* can be divided into two groups based on the width of the 2′′′′ plate (i.e. narrow or broad). *Gambierdiscus belizeanus* M.A. Faust, *Gambierdiscus australes* M. Chinain & M.A. Faust, *Gambierdiscus pacificus* M. Chinain & M.A. Faust, *Gambierdiscus excentricus* S. Fraga and *Gambierdiscus scabrosus* T. Nishimura, S. Sato & M. Adachi have narrow 2′′′′ plates, while *Gambierdiscus caribaeus* Vander-

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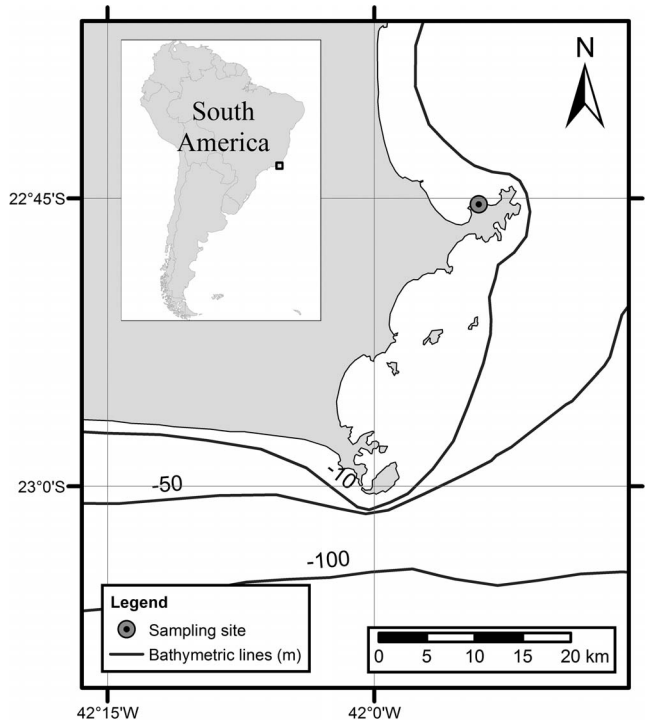


Fig. 1. The location of Armação dos Búzios at Rio de Janeiro, Brazil showing the place of strain isolation (sampling site).

sea, Litaker, Faust, Kibler, Holland & Tester; *Gambierdiscus carolinianus* Litaker, Vandersea, Faust, Kibler, Holland & Tester; *Gambierdiscus carpenteri* Kibler, Litaker, Faust, Holland, Vandersea & Tester; *Gambierdiscus polynesiensis* M. Chinain & M.A. Faust; *Gambierdiscus toxicus* R. Adachi & Y. Fukuyo and *Gambierdiscus silvae* S. Fraga & F. Rodríguez present broad 2′′′′ plates (Litaker *et al*. 2009; Fraga *et al*. 2011; Fraga & Rodríguez 2014; Nishimura *et al*. 2014). Several morphological and morphometric features were proposed to distinguish among *Gambierdiscus* species, including shape, size and symmetry of the 2′ and 2′′′′ plates as well as the pattern of the cell surface (Litaker *et al*. 2009).

The sulcal region in species of *Gambierdiscus* has been less studied as a result of the difficulties in visualizing the minute plates forming the innermost surface of the sulcus. This is because the sulcus forms a twisted clockwise hollow that has a three dimensional structure that is difficult to observe and to represent graphically. Morphological differences in thecal plate shape and size between species of *Gambierdiscus* are usually subtle and variable. The morphology of the sulcal plates might be a useful character to distinguish species as in the genus *Alexandrium* (Balech, 1995). In the few studies that considered the sulcal region, plate interpretation was variable between different authors. In the early descriptions of *Gambierdiscus* Adachi & Fukuyo (1979) and Taylor (1979) showed different sulcal plates with very different interpretations. Later studies on the thecae of *Gambierdiscus toxicus* (Loeblich & Indelicato 1986; Besada *et al*. 1982) did not describe the sulcal plates. The original descriptions of *Gambierdiscus belizeanus* (Faust 1995), *Gambierdiscus pacificus*, *Gambierdiscus australes*, *Gambierdiscus polynesiensis* (Chinain *et al*. 1999), *Gambierdiscus carpenteri* (Litaker *et al*. 2009) and *Gambierdiscus excentricus* (Fraga *et al*. 2011) did not include the morphology of sulcal plates. The only species whose sulcal plates were described and identified based on plate dissection were *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus* (Litaker *et al*. 2009) and *Gambierdiscus silvae* (Fraga & Rodríguez 2014). In this paper we describe the sulcal plates of *G. excentricus* based on material from the coast of Brazil. We review the *Gambierdiscus* sulcal area morphology and nomenclature used by different authors to name the sulcal plates in *Gambierdiscus*. Morphological description and molecular characterization of two *G. excentricus* strains isolated and cultured from the Southern Atlantic coastal waters are also presented.

MATERIALS AND METHODS

Macroalgal (*Sargassum furcatum* Kützing) samples were collected from a depth of 1–2 m by snorkel diving from Tartaruga Beach, Armação dos Búzios, Rio de Janeiro (22°45′18″ S, 41°54′07″ W, Fig. 1) in April 2013. Specimens of *S. furcatum* were placed in sealable plastic bags and vigorously shaken for 2 min to detach the associated epiphytic cells. An aliquot of the epiphytic suspension was preserved with neutral Lugol's iodine solution for observation of the morphology of *Gambierdiscus excentricus* field specimens. Live cells of *G. excentricus* were isolated from the epiphytic suspension using a micropipette and were sequentially transferred through four to five drops of sterile and filtered (glass-fibre filter, Millipore AP-40, Millipore Brazil) local seawater. After each transfer, the drop was examined to ensure a single cell was present. After the final transfer, each isolated cell was placed into a separate well of a sterile 96-well tissue culture plate with 90 µl of culture medium (Guillard's f/2 marine water enrichment solution, Sigma-Aldrich, St. Louis, MO, USA) prepared with local seawater which had been filtered (glass-fibre filter, Millipore AP-40, Millipore, São Paulo, Brazil), autoclaved, and the salinity adjusted to 32 with deionized water (dH_2O). When sufficient cell density was achieved, cells were transferred to a separate well of a sterile six-well tissue culture plate containing f/2 medium and were eventually transferred to 250 ml glass Erlenmeyer flasks. All stock cultures were maintained in a temperature-controlled cabinet at 24 ± 2°C, with a 12:12 h light:dark cycle and a photon flux density of 60 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Photosynthetically active radiation was measured with a QSL-100 quantum sensor (Biospherical Instruments, San Diego, CA, USA). The culture strains are available at the Rio de Janeiro State Federal University (UNIRIO) on request to S. Nascimento.

Lugol's fixed samples of strains UNR-07 and UNR-08 of *Gambierdiscus excentricus* were observed using a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast, differential interference contrast and epifluorescence optics, the latter using ultraviolet (UV) lamp HBO 100 W/2 (Osram GmbH, Munich, Germany) and a fluorescence filter cube with an excitation filter LP 425, a dichromatic filter 400 and an emission LP 425. For plate pattern identification, cells were stained with Fluorescent Brightener 28 (Sigma-Aldrich, St.

Louis, MO, USA) according to Fritz & Triemer (1985). Drops of diluted bleach were added to the slides under observation at the microscope to help to dissociate the sulcal plates. If necessary, dH_2O was added to the slide to replace evaporated volume. Cells were squashed by gently pressing the cover slip over them. Images were collected using an Axiocam high-resolution cooled digital camera and Zen image acquisition and analysis software (Zeiss, Oberkochen, Germany). When the depth of field was insufficient to capture the whole object, a series of pictures was taken at different focal planes, and these pictures were merged using Adobe Photoshop (CS6 (version 13.0×32, Adobe Systems Incorporated 1990–2012). Lugol preserved cells from the cultured strains and from field samples were stained with Fluorescent Brightener 28 (Sigma-Aldrich, St. Louis, MO, USA) and observed using epifluorescence microscopy with a UV lamp (upright Imager A.2, Zeiss) to measure cell size and the 2′/3′ and 2′/4′ plate's suture length. Images were collected using an AxiocamICc1 digital camera (Zeiss), and cells ($n = 30$) were measured using the Axiovision software (Zeiss). *Gambierdiscus excentricus* field specimens were identified based on cell morphology and the presence of a ventrally displaced apical pore plate.

Cultures of *Gambierdiscus excentricus* were fixed with neutral Lugol's iodine solution, filtered through 5 μm isopore membrane filters (EMD Millipore, Merck KGaA, Darmstadt, Germany), rinsed three times with distilled water and dehydrated in a series of 30, 50, 70, 80, 95, and 100% EtOH. After being air-dried overnight, they were coated with gold with a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a FEI Quanta 200 scanning electron microscope (FEI Company, Hillsboro, OR, USA) at the Centre for Scientific and Technological Support to Research, University of Vigo.

In this study, a modified Kofoid tabulation system (Kofoid 1909), as described in Besada *et al*. (1982), was followed to name the plates, and this enabled comparisons with other genera. Sulcal plates nomenclature was used as described by Balech (1995) for *Alexandrium* and Fraga & Rodríguez (2014) for *Gambierdiscus silvae*. The terms length (apical/antapical distance), width (transdiameter) and depth (dorso/ventral distance) were used to describe cell dimensions.

Exponentially growing cells of *Gambierdiscus excentricus* from strains UNR-07 and UNR-08 were harvested in 15 ml centrifuge tubes by centrifugation at $5000 \times g$ for 15 min for DNA extraction. Subsequently, the cells were transferred to 1.5 ml microtubes and centrifuged again at $5000 \times g$ for 15 min to settle the cells into pellets. Cell pellets were stored at –80°C for further analysis. Genomic DNA was extracted from the pellets using the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California, USA) following the manufacturer's instructions, and then stored at –20°C.

The D1–D3 and D8–D10 regions of the 28S rRNA gene (large subunit [LSU]) were amplified using two primers: D1R/LSUB (5′-ACCCGCTGAATTTAAGCATA-3′/5′-ACGAACGATTTGCACGTCAG-3′) (Scholin & Anderson 1994; Litaker *et al*. 2003) and FD8/RB (5′-GGATTGGCTCTGAGGGTTGGG-3′/5′-GATAGGAA-GAGCCGACATCGA-3′) (Chinain *et al*. 1999), respectively. The amplification reaction mixture (25 μl) contained 1 unit (U) Taq DNA polymerase (ThermoScientific Inc., USA), 1× reaction buffer with NH_4SO_4, 2.5 mM $MgCl_2$, 0.2 μg of bovine serum albumin BSA, 0.16 mM deoxyribonucleotide triphosphates (ThermoScientific Inc., Waltham, Massachusetts, USA) and 8 pmol of each primer. Conditions of the LSU gene amplification comprised an initial 5 min heating step at 95°C, followed by 40 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. A 5 μl aliquot of each polymerase chain reaction (PCR) was checked by electrophoresis in a 1% agarose gel stained with GelRed (Biotium Inc., Hayward, California, USA). Direct sequencing of the PCR products was performed by Macrogen Inc. (Macrogen Inc., Geumcheon-gu, Seoul, Korea) in an automated ABI Prism 3730 XL DNA analyzer (Applied Biosystems Inc., Waltham, Massachusetts, USA).

Individual consensus sequences were aligned using ClustalW implemented in MEGA6 (Tamura *et al*. 2013). All the detected polymorphisms were validated by visually checking the original electropherograms. Consensus sequences were searched with the basic local alignment search tool against the GenBank database (www.ncbi.nlm.nih.gov/blast). GenBank accession numbers for the used sequences are provided in Figs 2 and 3. Phylogenetic analyses were performed separately for the LSU D1–D3 and D8–D10 loci. Phylogenetic trees were reconstructed using the maximum likelihood (ML) analysis with 1000 bootstrap replications implemented in MEGA6 (Tamura *et al*. 2013) and the Bayesian inference (BI) method implemented in MrBayes 3.2.2 (Ronquist *et al*. 2012) exactly as described by Fraga & Rodríguez (2014). The LSU D1–D3 and D8–D10 sequences obtained in this study were deposited in GenBank (KP290886, KP290887, KP290888, KP290889).

RESULTS

Phylogeny of *Gambierdiscus excentricus* strains from Rio de Janeiro

The final alignment of the D1–D3 region based on 92 sequences included 1054 nucleotides with 702 variable sites. Additionally, the alignment of the D8–D10 region comparing 155 sequences included 799 nucleotides with 422 variable sites.

The D1–D3 and D8–D10 LSU phylogenies of *Gambierdiscus* species including the two *Gambierdiscus excentricus* strains (UNR-07 and UNR-08) from Brazil are shown in Figs 2 and 3. The two strains clustered together with the sequences from strains of *G. excentricus* from the Canary Islands, its type locality, in both D1–D3 and D8–D10 analyses.

Morphology

The cells were lenticular in shape (Figs 4–7), and the thecal surface was smooth with evenly distributed round to oval pores (Figs 4–8, 9–10, 14–15). The plate formula was Po, 4′, 0a, 6″, 6c, 8s?, 5‴, 0p, 2⁗. The depth of cultured cells was 74 ± 6 μm (60–95 μm), and the width was 70 ± 7 μm (53–91 μm) (means of the two cultured strains, dimensions not significantly different). Cells from field samples had an average depth of 93 ± 9 μm (62–107 μm) and average width of 84 ± 9

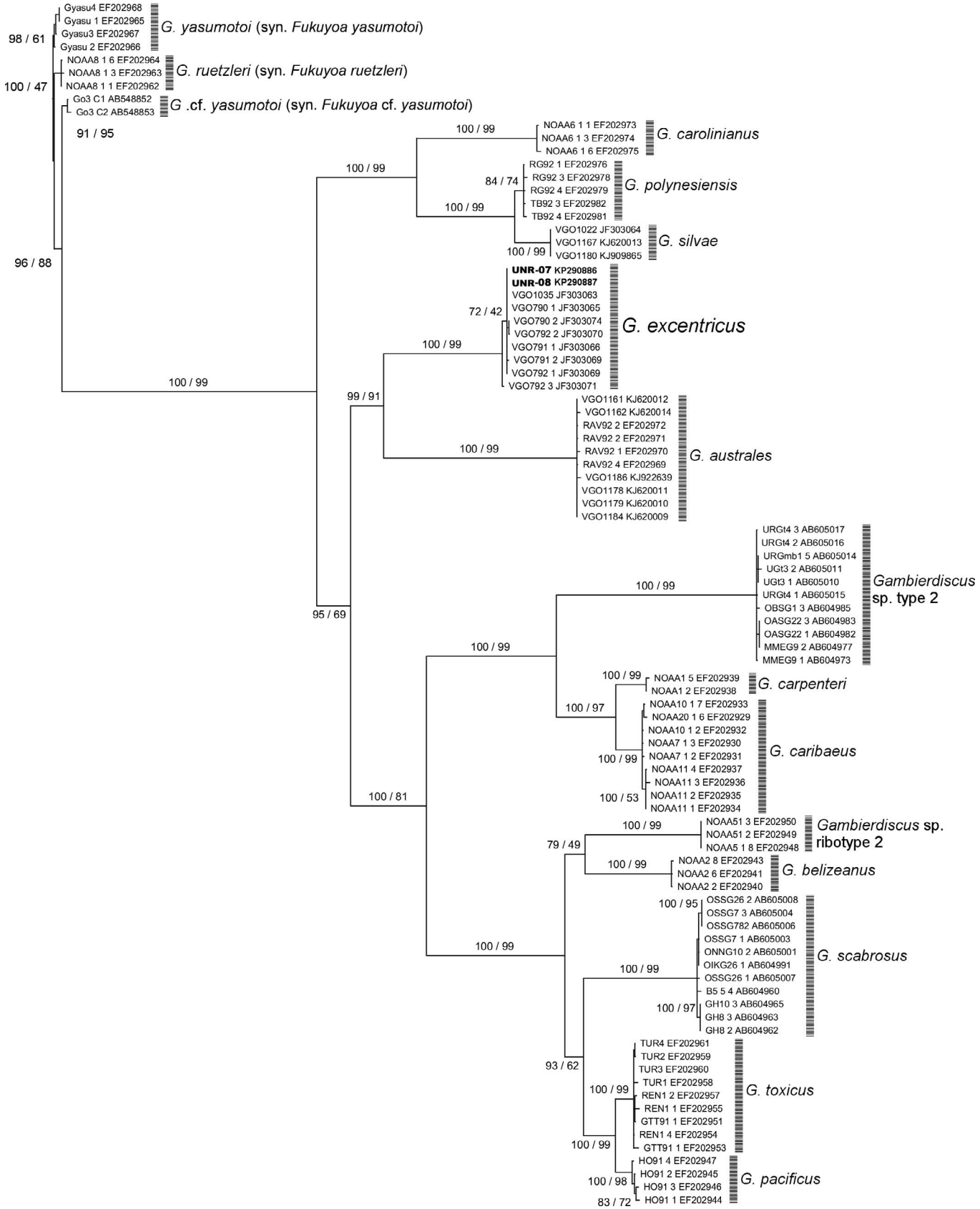
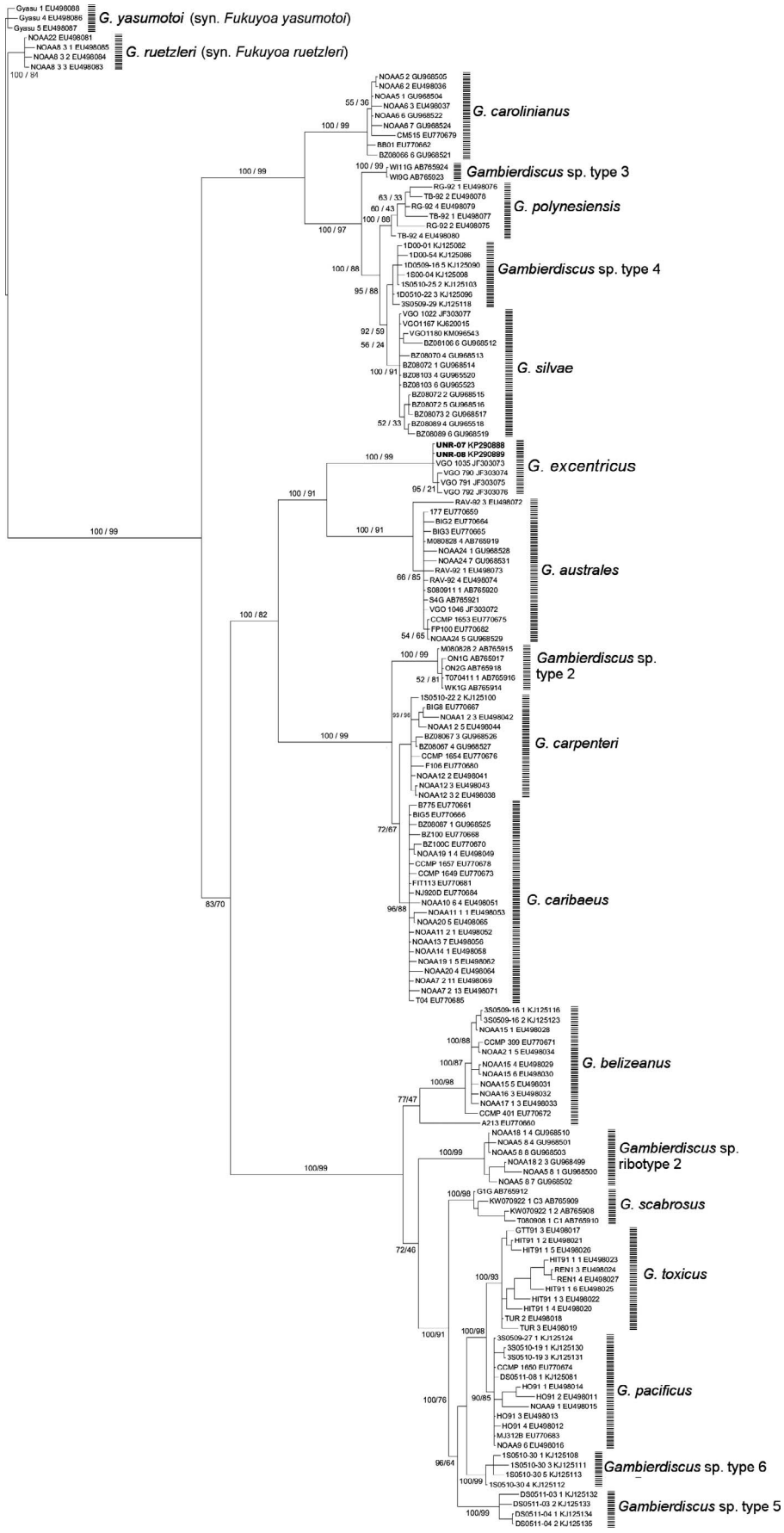


Fig. 2. Bayesian inference (BI) phylogeny of the LSUrRNA D1–D3 region of *Gambierdiscus* species/phylotypes. Phylogenetic tree presenting consensus topology from Bayesian and maximum likelihood (ML) analysis. New sequences published in this study are displayed in bold (UNR-07 <KP290886> and UNR-08 <KP290887>). Strains are identified by their name and GenBank accession number. Supports at internal nodes are Bayesian posterior probability (Bayesian analysis) and bootstrap support values from ML analysis, respectively.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Gyasu 4 EU498086
Gyasu 5 EU498087
G. yasumotoi (syn. Fukuyoa yasumotoi)
NOAA22 EU498081
NOAA8 3 1 EU498085
NOAA8 3 2 EU498084
NOAA8 3 3 EU498083
G. ruetzleri (syn. Fukuyoa ruetzleri)
100 / 94
NOAA5 2 GU968505
NOAA6 2 EU498036
NOAA5 1 GU968504
55 / 36
NOAA6 3 EU498037
NOAA6 6 GU968522
NOAA8 7 GU968524
CM515 EU770679
BB01 EU770662
BZ06066 6 GU968521
G. carolinianus
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100 / 99
WI11G AB765924
WI9G AB765923
Gambierdiscus sp. type 3
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RG-92 1 EU498076
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TB-92 2 EU498078
RG-92 4 EU498079
60 / 43
TB-92 1 EU498077
100 / 88
RG-92 2 EU498075
100 / 97
TB-92 4 EU498080
G. polynesiensis
1D00-01 KJ125082
1D00-54 KJ125086
100 / 88
1D0509-16 5 KJ125090
1S00-04 KJ125008
95 / 88
1S0510-25 2 KJ125103
1D0510-22 3 KJ125096
3S0509-29 KJ125118
Gambierdiscus sp. type 4
VGO 1022 JF303077
VGO1167 KJ620015
92 / 59
VGO1180 KM096543
56 / 24
BZ08106 6 GU968512
BZ08070 4 GU968513
BZ08072 1 GU968514
100 / 91
BZ08103 4 GU965520
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BZ08073 2 GU968517
BZ08089 4 GU965518
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G. silvae
UNR-07 KP290888
UNR-08 KP290889
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VGO 791 JF303075
VGO 792 JF303076
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Gambierdiscus sp. ribotype 2
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T080908 1 C1 AB765910
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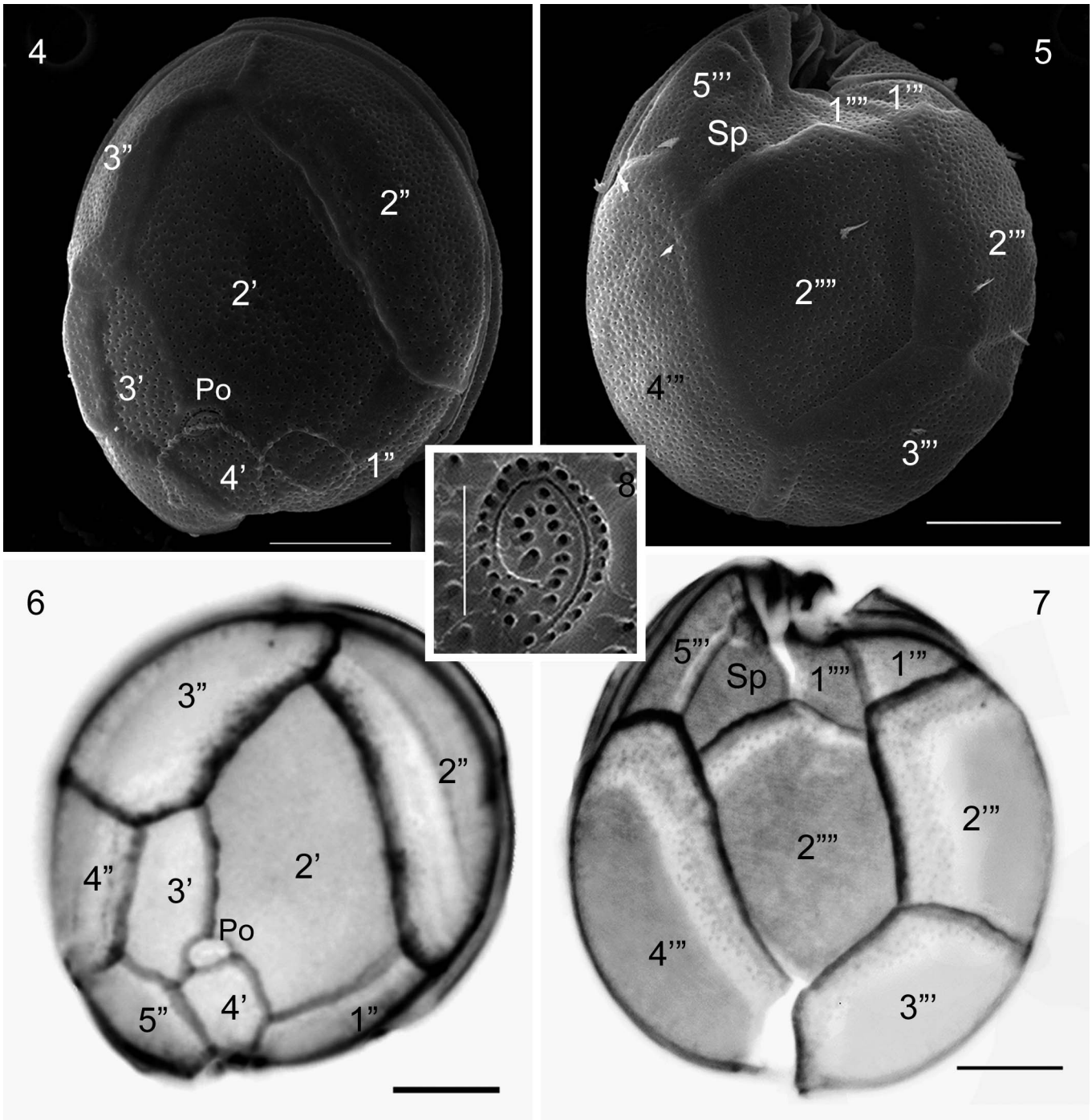

Figs 4–8. Scanning electron mecroscope (SEM) micrographs and Fluorescent Brightener 28-stained cells of *Gambierdiscus excentricus*.
Fig. 4. SEM micrograph of *Gambierdiscus excentricus* in apical view. Scale bar = 20 μm.
Fig. 5. SEM micrograph of *Gambierdiscus excentricus* in antapical view. Scale bar = 20 μm.
Fig. 6. Fluorescent brightener 28-stained *Gambierdiscus excentricus* cell in apical view. Scale bar = 20 μm.
Fig. 7. Fluorescent brightener 28-stained *Gambierdiscus excentricus* cell in antapical view. Scale bar = 20 μm.
Fig. 8. SEM micrograph of the apical pore plate (Po). Scale bar = 5 μm.

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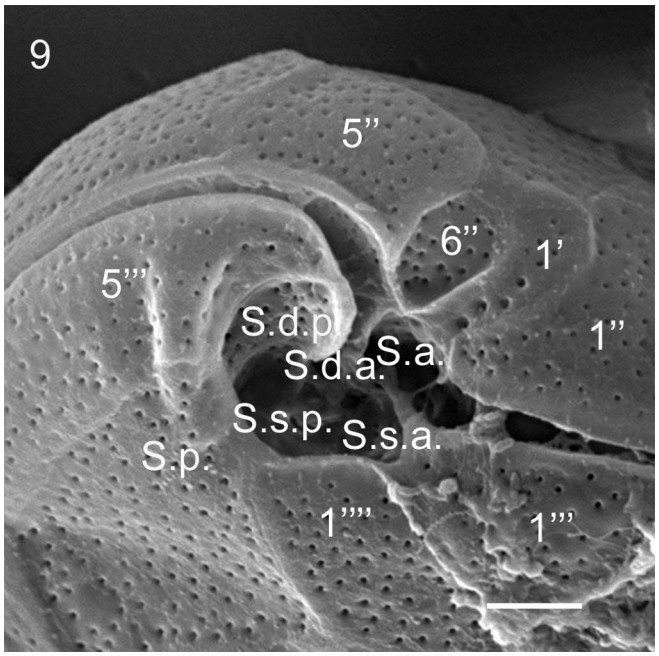
Fig. 3. Bayesian inference (BI) phylogeny of the LSUrRNA D8–D10 region of *Gambierdiscus* species/phylotypes. Phylogenetic tree presenting consensus topology from Bayesian and maximum likelihood (ML) analysis. New sequences published in this study are displayed in bold (UNR-07 <KP290888> and UNR-08 <KP290889>). Strains are identified by their name and GenBank accession number. Supports at internal nodes are Bayesian posterior probability (Bayesian analysis) and bootstrap support values from ML analysis, respectively.



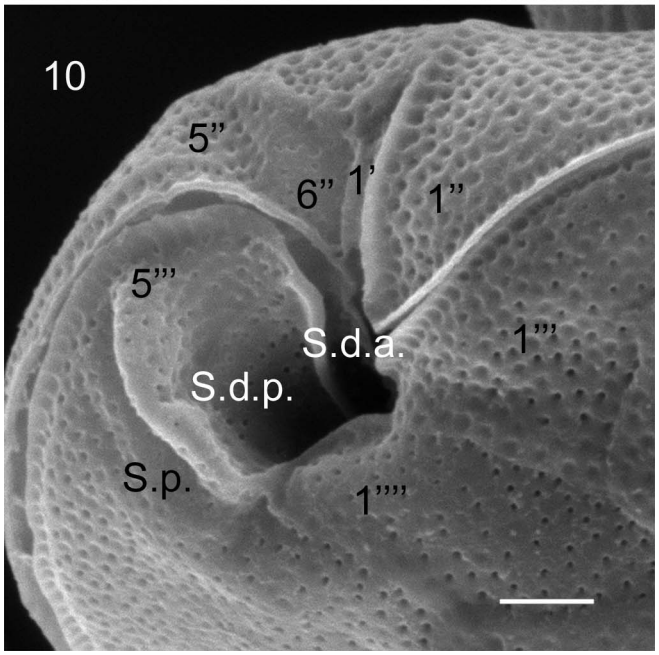


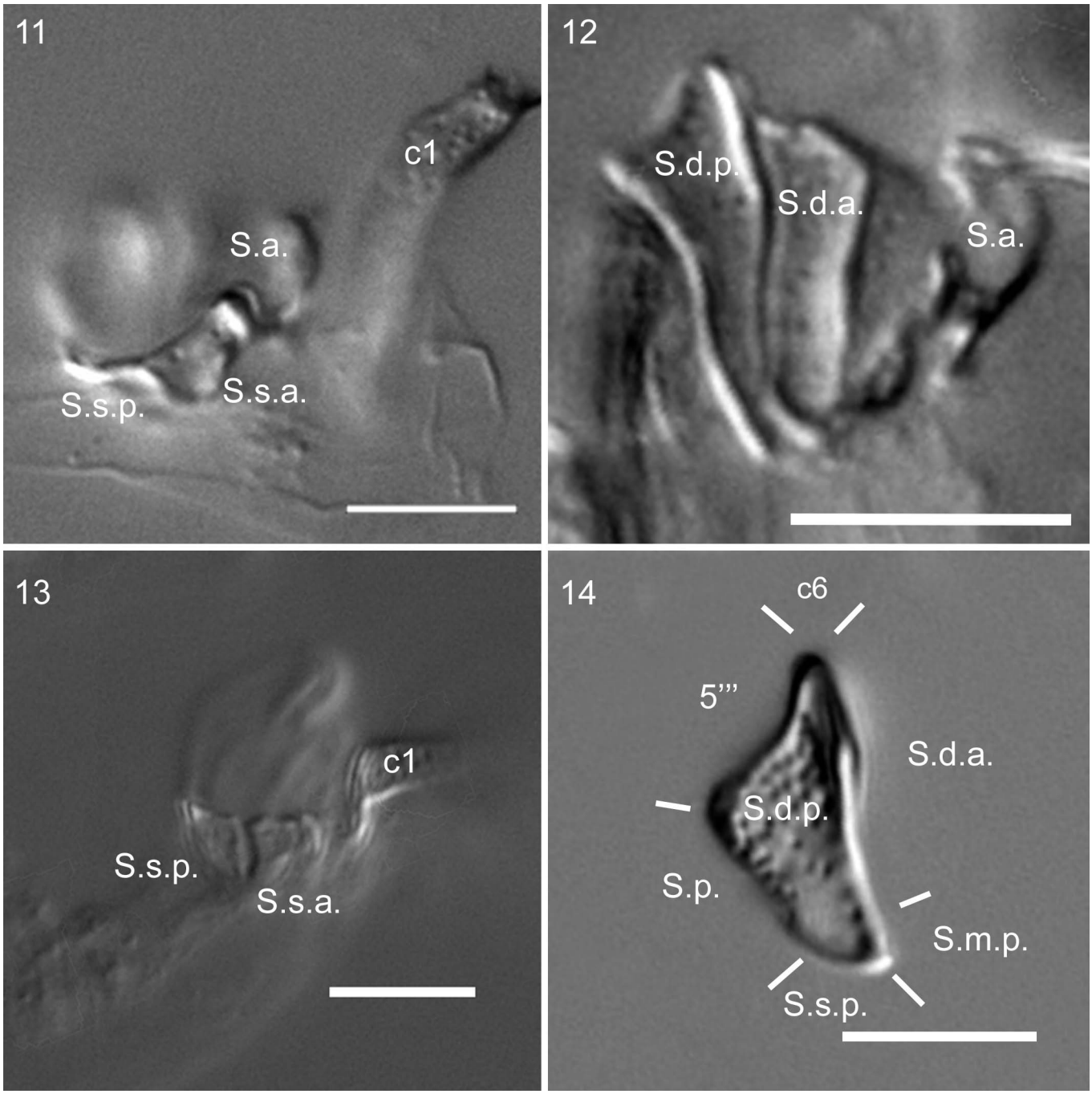
Fig. 9. SEM micrograph of *Gambierdiscus excentricus* sulcus showing the locations of the 1′, 1″, 5″, 6″, 1‴, 5‴, 1⁗, S.d.p., S.d.a., S.a., S.s.p., S.s.a. and S.p. plates in an intact cell. Scale bar = 5 μm.

Fig. 10. SEM micrograph of *Gambierdiscus excentricus* sulcus showing the position of the S.d.p. and S.d.a. plates in more detail. Scale bar = 5 μm.

μm (59–100 μm). The apical pore plate Po was oval with a fishhook-shaped slit surrounded by a row of pores (Fig. 8) and was displaced ventrally (Figs 4, 6). As a consequence of the displacement, the ratio between the 2′/3′ and 2′/4′ suture length was, on average, 2.2 in cultured cells and 2.6 in field specimens. Plate 1′ was very small and narrow (Fig. 9) and was not visible in apical view (Figs 4, 6). It did not contact Po and was compressed by plates 1″ and 6″ (Fig. 9). The second apical plate (2′) was the largest of the apical series (Figs 4, 6). It was more or less rectangular and dorsally pointed (Figs 4, 6). Plates 2″ and 3″ were the largest of the precingular series and occupied the entire dorsal portion of that series (Figs 4, 6). The cingulum was descendent about one girdle width; although, in ventral view it appeared to be ascendant due to a clockwise torsion of the ventral area (Figs 9–10). The cingulum was composed of six plates, and c1 and c6 were curved as a result of the torsion of that area (Figs 9–10).

The hypotheca was composed of five postcingular plates, two antapical plates and the sulcal posterior (S.p.) (Figs 5, 7). In the postcingular series the four sided 4‴ was the largest plate and occupied most of the right side of the hypotheca (Figs 5, 7). Triangular 1‴ and curved 5‴ plates were smaller in this series (Figs 5, 7). The edge of the 5‴ adjacent to the sulcus was twisted, accompanying the torsion of the ventral area of the cell (Figs 9–10). The sulcal posterior was situated outside of the sulcus and was displaced to the right side of the hypotheca as a result of the torsion of the ventral area (Figs 5, 7, 9–10). In the antapical series, 1⁗ was broadly symmetrical to S.p. (Figs 5, 7, 9). The 2⁗ plate was pentagonal and slightly wider at the ventral side (Figs 5, 7). Many atypical cells were observed among the cultures, including substantial plate overlap, particularly in the hypothecal plates.

The sulcus of *Gambierdiscus excentricus* was deep, with a recessed pouch shape that formed a two-chambered hollow (Figs 9–10). Detailed observation of the sulcus was carried out in cells from strains UNR-07 and UNR-08. The anterior chamber was composed of the right sulcal posterior (S.d.p.), right sulcal anterior (S.d.a.) and sulcal anterior (S.a.) plates (Figs 9–17). The posterior chamber was comprised of the left anterior lateral (S.s.a.) and the left posterior lateral (S.s.p.) plates (Figs 9, 11, 13). The S.p. plate was identified as a sulcal plate; although in *Gambierdiscus* it is located outside the sulcus. At the anterior chamber, the S.d.p. plate was triangular with one end twisted and forming a thickened ridge along the juncture with the cingulum (Figs 9–10, 12, 14–17). The S.d.a. plate was narrow and long and was laterally positioned between the S.d.p. and S.a. (Figs 9–10, 12, 15–17). The S.d.a. is considered by many authors to be the 't' or transitional plate. The S.d.p. and S.d.a. had sharper ends toward the base of the sulcus (Figs 12, 14–17) that protruded deeply into the cell. The S.d.a. extended from the sixth cingular plate until the bottom of the sulcal pocket (Figs 9–10, 12, 15–17). The S.a. plate was positioned beside the S.d.a. plate (Figs 9, 12, 15–17) and was taller at its central portion and cup-shaped (Figs 9, 12, 15–17). The S.d.p., S.d.a., and S.a. plates typically remained attached when the sulcus was dissociated, and the S.a. plate was observed laterally most of the time. These plates were larger than the S.s.a. and S.s.p. plates (Figs 11, 13) that were located at the posterior chamber of the sulcus, behind the S.p. The surface of the sulcal plates were smooth with pores (Figs 9–10, 14–15). The hollow of the sulcal area was limited at the posterior chamber by the anterior edges of the 5‴, S.p., 1⁗ and the lateral portion of plate 1‴ (Figs 9–10). Two small plates, the anterior medium plate (S.m.a.) and posterior medium plate (S.m.p.), which were located at the juncture of the S.d.p., S.d.a., S.a., S.s.a. and S.s.p. plates were also observed (Figs 15–17). The sulcal region of *G. excentricus* was



Figs 11–14. Light micrographs of dissected *Gambierdiscus excentricus* sulcal plates (see also Figs 9–10 for the location of the corresponding plates in intact cells).

Fig. 11. The hollow-shaped sulcus showing the location of sulcal plates. S.s.p. and S.s.a. plates in focus and the outline of the anterior chamber with the S.d.p., S.d.a. and S.a. plates discernible. Scale bar = 10 μm.

Fig. 12. Dissected sulcal plates showing the anterior chamber with the S.d.p., S.d.a. and S.a. plates. Scale bar = 10 μm.

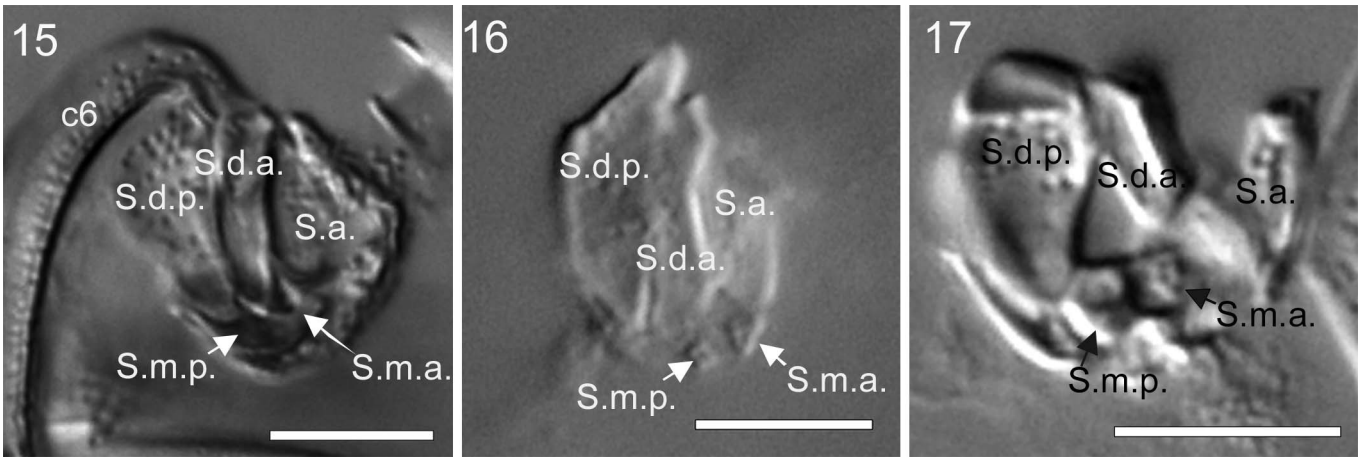
Fig. 13. The posterior sulcal chamber with the S.s.p. and S.s.a. plates clearly visible. Scale bar = 10 μm.

Fig. 14. Expanded view of the S.d.p. plate showing its characteristic shape and indication of the position of neighbouring plates. Scale bar = 10 μm.

twisted to the right and protruded into the cell. The posterior chamber of the sulcus was folded such that plates S.s.a. and S.s.p. were positioned behind the 1′′′′ and the S.p. plates (Figs 18–20).

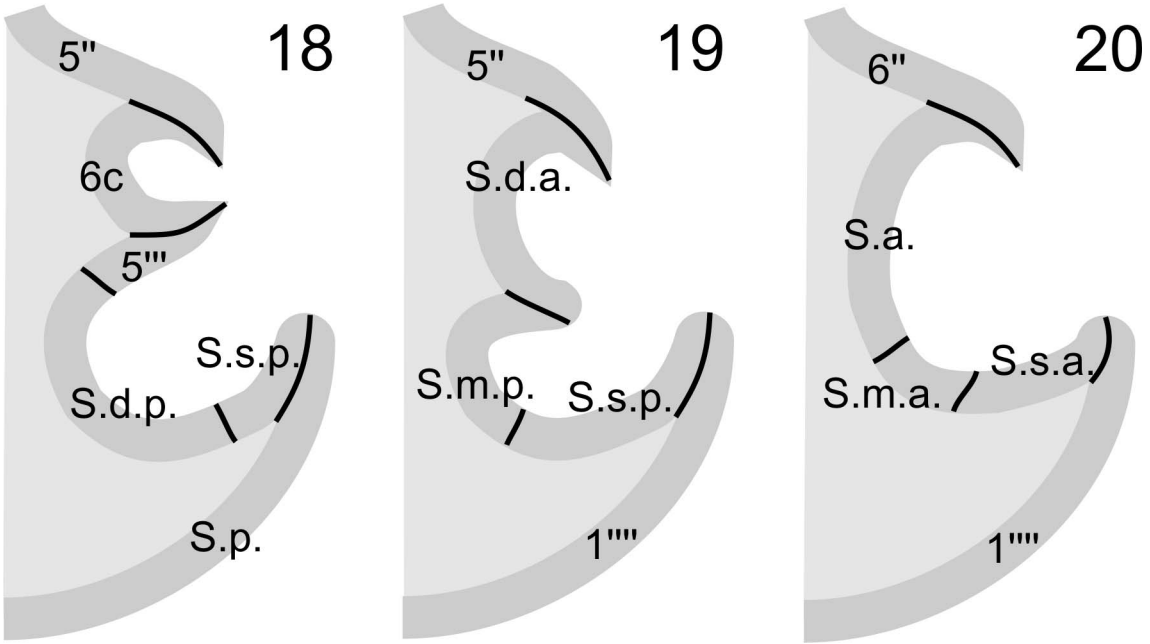
DISCUSSION

The phylogenetic relationships (LSU rRNA) between species of *Gambierdiscus* and phylotypes in our study were similar to



Figs 15–17. Light micrographs of dissected sulcal plates from cleared *Gambierdiscus excentricus* cells in expanded view.
Fig. 15. The anterior chamber of the sulcus and the location where the sixth cingular plate (c6) reaches the S.d.a. plate. The position of the minute S.m.p. and S.m.a. plates are indicated. Scale bar = 10 μm.
Fig. 16. Dissected sulcus showing the architecture of the S.d.p., S.d.a. and S.a. plates at the anterior chamber and the S.m.p. and S.m.a. plates that reside at the lower part of the sulcus. Scale bar = 10 μm.
Fig. 17. Dissected sulcus with the S.m.p. and S.m.a. plates visible. In this micrograph the S.d.a. plate has been slightly displaced from its normal position. Scale bar = 10 μm.

those reported by other authors (Litaker *et al*. 2010; Fraga *et al*. 2011; Nishimura *et al*. 2013; Xu *et al*. 2014; Fraga & Rodríguez 2014). The morphology of the strains of *Gambierdiscus excentricus* and cells from field populations was consistent with that described by Fraga *et al*. (2011), with a ventrally displaced Po and, as a result, a high ratio between the 2′/3′ and 2′/4′ suture lengths. While in *G. excentricus* this ratio is around 2.3 for the Canary Island cells and 2.4 for the cells from Brazil (mean of cultured and field specimens), this ratio ranges between 1.0 and 1.6 in all other species in the genus (Fraga *et al*. 2011; Nishimura *et al*. 2014). This is a unique morphological characteristic among all the known species of *Gambierdiscus* (Fraga *et al*. 2011). Cell dimensions of strains of *G. excentricus* fit within the lower end of the size range reported for specimens from the Canary Islands by Fraga *et al*. (2011) and included cells with smaller dimensions. Cells from the field samples were generally larger than the cultured cells. Several specimens



Figs 18–20. Line drawings showing the position of sulcal plates and adjacent plates across schematic vertical cuts along the sulcal area from right, middle to the left side of the sulcus of *Gambierdiscus excentricus*.
Fig. 18. Vertical cut showing the right end of the sulcal area.
Fig. 19. Vertical cut across the middle region of the sulcal area.
Fig. 20. Vertical cut showing the left end of the sulcal area.

of *G. excentricus* with aberrant plate patterns were observed among cultured cells, as has been reported for other species such as *Fukuyoa yasumotoi* (as *Gambierdiscus yasumotoi*) (Saburova *et al.* 2013).

The sulcal plates of *Gambierdiscus* species are difficult to visualize because the sulcus is twisted and depressed into the cell, forming a deep, tight funnel. As reviewed by Litaker *et al.* (2009) the number of plates comprising the sulcal series in *Gambierdiscus* has been variously described as eight (Adachi & Fukuyo 1979; Loeblich & Indelicato 1986), presumably eight (Faust 1995; Chinain *et al.* 1999; although a figure of the sulcal area is not shown in these papers), and seven (Litaker *et al.* 2009, who did not consider the S.p. plate to be a part of the sulcus, as it lies outside of the sulcal pocket and in the same plane as the 1‴ plate). This variability in the number of sulcal plates arises from the difficulties in visualizing the minute plates forming the innermost surface of the sulcus. Moreover, different authors use diverse nomenclatures to name the plates (Table S1, Supplementary Data). For example, the plate to the right of the 1′′′′, named S.p. in the current study following Besada *et al.* (1982), Loeblich & Indelicato (1986), Fensome *et al.* (1993), Fraga *et al.* (2011), Nishimura *et al.* (2014) and Fraga & Rodríguez (2014), is considered by some authors to be the 2′′′′ in the antapical series (Faust 1995; Chinain *et al.* 1999; Litaker *et al.* 2009) or 3′′′′ (Taylor 1979), i.e. not a part of the sulcal series.

The sulcal plate arrangement of *Gambierdiscus* has been reported from *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri*, *Gambierdiscus toxicus* (Litaker *et al.* 2009), *Gambierdiscus caribaeus* (Litaker *et al.* 2009; Jeong *et al.* 2012) and *Gambierdiscus silvae* (Fraga & Rodríguez 2014). Litaker *et al.* (2009) described the sulcal region from the *Gambierdiscus* species as including the S.d.p., the transition (t) plate (here named S.d.a.), S.d.a. (here named S.a.), S.s.p. and S.s.a. Those authors also reported the presence of an S.m. plate (and equivalent S.m.a. and S.m.p. plates in the related species *Fukuyoa ruetzleri* and *Fukuyoa yasumotoi*, as *Gambierdiscus ruetzleri* and *Gambierdiscus yasumotoi*), which forms the dorsal end of the sulcal pocket.

The general arrangement of the sulcal plates of *Gambierdiscus excentricus* is similar to the other *Gambierdiscus* species that have been published (e.g., Litaker *et al.* 2009; Fraga & Rodríguez 2014) in relation to the S.d.p., S.d.a. and S.a. plates that comprise the anterior chamber of the sulcal pocket and the S.s.p. and S.s.a. on the opposite posterior chamber. Loeblich & Indelicato (1986) show a light micrograph of the *Gambierdiscus toxicus* sulcus where these plates can be easily recognized as well. Two small plates (S.m.a. and S.m.p.) located at the juncture of the S.d.p., S.d.a., S.a., S.s.a. and S.s.p. plates were shown for the first time in the current study. Loeblich & Indelicato (1986) describe the sulcus of *G. toxicus* as composed of six large plates and at least two smaller internal plates, which are probably the plates S.m.a. and S.m.p. In the original description of the genus, Adachi & Fukuyo (1979) also reported two small median plates but indicated that they are above rather than below the S.a. plate.

The S.m.a. and S.m.p. plates are difficult to detect because of their small size and the sulcus structure in *Gambierdiscus* species. Moreover, the posterior chamber of the sulcus is folded so that the S.s.p. and S.s.a. are positioned behind the S.p. and the 1′′′′ plate (see Figs 18–20). In contrast, in *Alexandrium* species, a membranous portion supports both the S.d.p. and S.s.p. below the plane of the S.p. (Balech 1995).

Among the sulcal plates, the S.d.p. is more easily observed than the others. In *Gambierdiscus excentricus,* this plate was triangular with a reinforced and folded side that accompanied the cingular list. Comparing the S.d.p. of *G. excentricus* with the S.d.p. of *Gambierdiscus carolinianus* and *Gambierdiscus caribaeus* shown in Litaker *et al.* (2009) and the plates shown in Loeblich & Indelicato (1986), it is clear that the shape of this plate differs among *Gambierdiscus* species. The S.d.p. of *G. excentricus* was narrower, particularly toward its posterior end, than the S.d.p. of *G. carolinianus* and *G. caribaeus*. In species of *Alexandrium* the sulcal plates have taxonomic value, particularly the S.a. and S.p. plates where the most distinctive characteristics are found (Balech 1995). The S.a. plate of *G. excentricus* had a long central portion and was roughly comparable to the S.a. plate with a precingular section found in a few species of *Alexandrium*, such as *Alexandrium tamiyavanichii* Balech. To date, the sulcal plates of only a few *Gambierdiscus* species have been observed, and their variability among species is unknown. As occurred in *Alexandrium*, more information about the sulcal plates of *Gambierdiscus* may provide valuable information to help distinguish among species.

In this paper, we have expanded knowledge of the distribution of *Gambierdiscus* species in the South Atlantic. To date, some species were only found in the Atlantic or Pacific Oceans but, as predicted (Litaker *et al.* 2010), more geographically intense sampling showed broader distributions than originally determined. For example, *Gambierdiscus australes* was reported previously only in the Pacific (Litaker *et al.* 2010) but has recently been found in the Atlantic (Fraga & Rodríguez 2014). In addition, *Gambierdiscus belizeanus*, reported previously from the Caribbean Sea (Litaker *et al.* 2010), was recently identified from Malaysia and Jordan (Leaw *et al.* 2011; Saburova *et al.* 2013). Xu *et al.* (2014) reported three new *Gambierdiscus* ribotypes, in addition to *Gambierdiscus carpenteri*, *Gambierdiscus pacificus* and *G. belizeanus*, from the Republic of Kiribati in the Central Pacific. This further broadens the distribution of *G. belizeanus* to Micronesia. *Gambierdiscus carpenteri* was recently reported from Australia, in high abundance at a site on the south-east coast (Kohli *et al.* 2014). *Gambierdiscus polynesiensis*, *G. pacificus* and *G. australes* were also reported from the Cook Islands (Rhodes *et al.* 2014). The recently described species, *Gambierdiscus silvae*, was documented from the Canary Islands and as ribotype-1 from the Caribbean Sea (Litaker *et al.* 2010).

Previously, *Gambierdiscus excentricus* was only reported from the Canary Islands, i.e. the type locality for the species (Fraga *et al.* 2011), and also on the coasts of Morocco (Fraga *et al.* 2011; Ennaffah & Chaira 2015) and Oman (Maria Saburova, personal communication). It was not observed in the Caribbean Sea, despite intensive studies to assess *Gambierdiscus* diversity that have identified five species in the area (Litaker *et al.* 2010; Vandersea *et al.* 2012).

The Brazilian coast appears to be free of ciguatera, with no published reports of cases but it is possible that mild cases may have been overlooked. The coral and algal reefs of the south-east and north-east coastal areas of Brazil provide extensive favourable habitat for epibenthic dinoflagellates, i.e. the environmental conditions known to sustain *Gambierdiscus* (Litaker *et al*. 2010) and other epibenthic dinoflagellate species. These conditions include annual seawater temperature between 21 and 31°C, abundant macrophytes, low to moderate turbulence, high and stable salinity, actual or attenuated light levels and sufficient nutrient concentrations (Litaker *et al*. 2010, and references therein).

In the original description of *Gambierdiscus excentricus*, the cells were toxic to Neuro-2a cells, which indicates the production of ciguatoxin and maitotoxin (Fraga *et al*. 2011). There is no monitoring program for *Gambierdiscus* and ciguatoxins in fish flesh along the Brazilian coast, and the diversity and abundance of *Gambierdiscus* species is largely unknown. The risk of ciguatera in a particular area is considered proportional to the cell abundance of *Gambierdiscus* (Litaker *et al*. 2010). However, the risk is also strongly dependent on the species of *Gambierdiscus* present in that area, as each species of *Gambierdiscus* has a unique toxin profile (Chinain *et al*. 2010; Kohli *et al*. 2014). Further studies are necessary to assess species diversity and abundance of *Gambierdiscus* in the region. Where species of *Gambierdiscus* are detected, more than one species is often present (Litaker *et al*. 2010; Fraga & Rodríguez 2014).

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/15-61.1.s1.

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