Marine Biodiversity Records

MARINE RECORD Open Access

Morphological and molecular phylogenetic identification and record verification of *Gambierdiscus excentricus* (Dinophyceae) from Madeira Island (NE Atlantic Ocean)

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Abstract

The marine benthic dinoflagellate genus *Gambierdiscus* currently contains ~ 16 species that can be highly morphologically similar to one another, and therefore molecular genetic characterization is necessary to complement the morphological species determination. *Gambierdiscus* species can produce ciguatoxins, which can accumulate through the food chain and cause ciguatera fish poisoning. Recent studies have suggested that *Gambierdiscus excentricus* may be one of the main species responsible for ciguatoxin production in the temperate and tropical regions of the eastern Atlantic. The present study definitively identifies the species, *G. excentricus*, from Madeira Island, Northeast-Atlantic Ocean (32° 38′ N 16° 56′ W) by examining the morphology of a strain using light and scanning electron microscopy and sequencing regions of the ribosomal DNA (D8-D10 LSU, SSU rDNA). Variability in the shape of the apical pore and the microarchitecture of the apical pore plate were documented for the first time, as well as variability in the width of the second antapical plate. The first SSU rDNA sequence for the species is reported. Because *G. excentricus* is known to produce high levels of CTX-like compounds, its presence and toxicity should be regularly monitored to establish whether it is the primary cause of the ciguatera poisoning events on Madeira Island.

Keywords: Benthic, Epiphytic, *Gambierdiscus*, Morphology, Phylogeny, SSU rDNA

Background

The marine benthic dinoflagellate genus *Gambierdiscus* was discovered in the late 1970s (Yasumoto et al., 1977) and described with *G. toxicus* R.Adachi & Y.Fukuyo as the type species (Adachi & Fukuyo, 1979). Currently ~ 16 species of *Gambierdiscus* have been described, based on their distinct morphological and genetic characteristics (Adachi & Fukuyo, 1979; Chinain et al., 1999; Faust, 1995; Fraga et al., 2011; Litaker et al., 2009; Litaker et al., 2010; Nishimura et al., 2014; Fraga & Rodriguez, 2014; Fraga et al., 2016; Smith et al., 2016; Kretzschmar et al., 2017; Rhodes et al., 2017). The genus *Fukuyoa* F.Gómez, D.Qiu, R.M.Lopes & S.Lin was separated from *Gambierdiscus* in 2015, and now includes three species (Gómez et al., 2015). Some species can show intra-specific morphological variability (Bravo et al., 2014). Others can be highly morphologically similar to one another (e.g., Kretzschmar et al., 2017; Kohli et al., 2014a). Molecular genetic characterization is necessary to complement the morphological species determination. The genus lives epiphytically on many substrates in shallow tropical and sub-tropical waters (Hoppenrath et al., 2014 and references therein). Certain *Gambierdiscus* species, most conspicuously *G. polynesiensis*, are the primary sources of ciguatoxins (CTXs), the causative agent of ciguatera fish poisoning (CFP) (e.g., Chinain et al., 1999; Chinain et al., 2010; Holmes, 1998; Berdalet et al., 2012). Maitotoxins (MTXs) are also commonly produced (Kohli et al., 2014b). *Fukuyoa* species can also produce MTXs (Rhodes et al., 2014).

Gambierdiscus excentricus S.Fraga was described as a new species in 2011 from Tenerife, Canary Islands (Spain) and was also recorded from La Gomera and La

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Palma (Fraga et al., 2011). A characteristic feature of *G. excentricus* is the ventrally displaced apical pore complex (APC) resulting in a relatively long suture between the second (2′) and third (3′) apical plate, and the hypotheca has a narrow second antapical plate 2′′′′ (Fraga et al., 2011). The molecular definition of the species was based on partial large subunit ribosomal DNA (LSU rDNA) sequences (Fraga et al., 2011). In 2015 details of the sulcal morphology, gained from a Brazilian strain, completed the species description (Nascimento et al., 2015). The toxin profile using a neuroblastoma cell-based assay indicated high ciguatoxic (CTX) and maitotoxic (MTX) activity (Fraga et al., 2011; Pisapia et al., 2017). Further Pisapia et al. identified that some strains produced a novel toxin analogue, named MTX4 (Pisapia et al., 2017). To date, the profile of CTX toxins of *G. excentricus* has not been confirmed with LC-MS/MS.

Madeira Island is located about 870 km from the Iberian Peninsula, 700 km from the West African coast (Fig. 1) and between 450 to 550 km from the Canary Islands. It is the main island of an archipelago comprising 4 other islands nearby (Porto Santo about 45 km to the northeast and the 3 Desertas islands about 20 km to the southeast). About 300 km to the south are the 2 islands of Selvagens archipelago, also belonging to Madeira. The islands are situated at the eastern boundary of the North Atlantic Subtropical Gyre with dominating northeastern trade winds and typical oligotrophic conditions (Longhurst et al., 1995). The first harmful algal species were recorded on Madeira in 2002, and in 2007 the first case of ciguatera fish poisoning was reported for the Selvagens islands (Otero et al., 2010, and references therein). More cases occurred in subsequent years with verification of CTXs involved in CFP (Otero et al., 2010). Monitoring of possible CTX-producing species

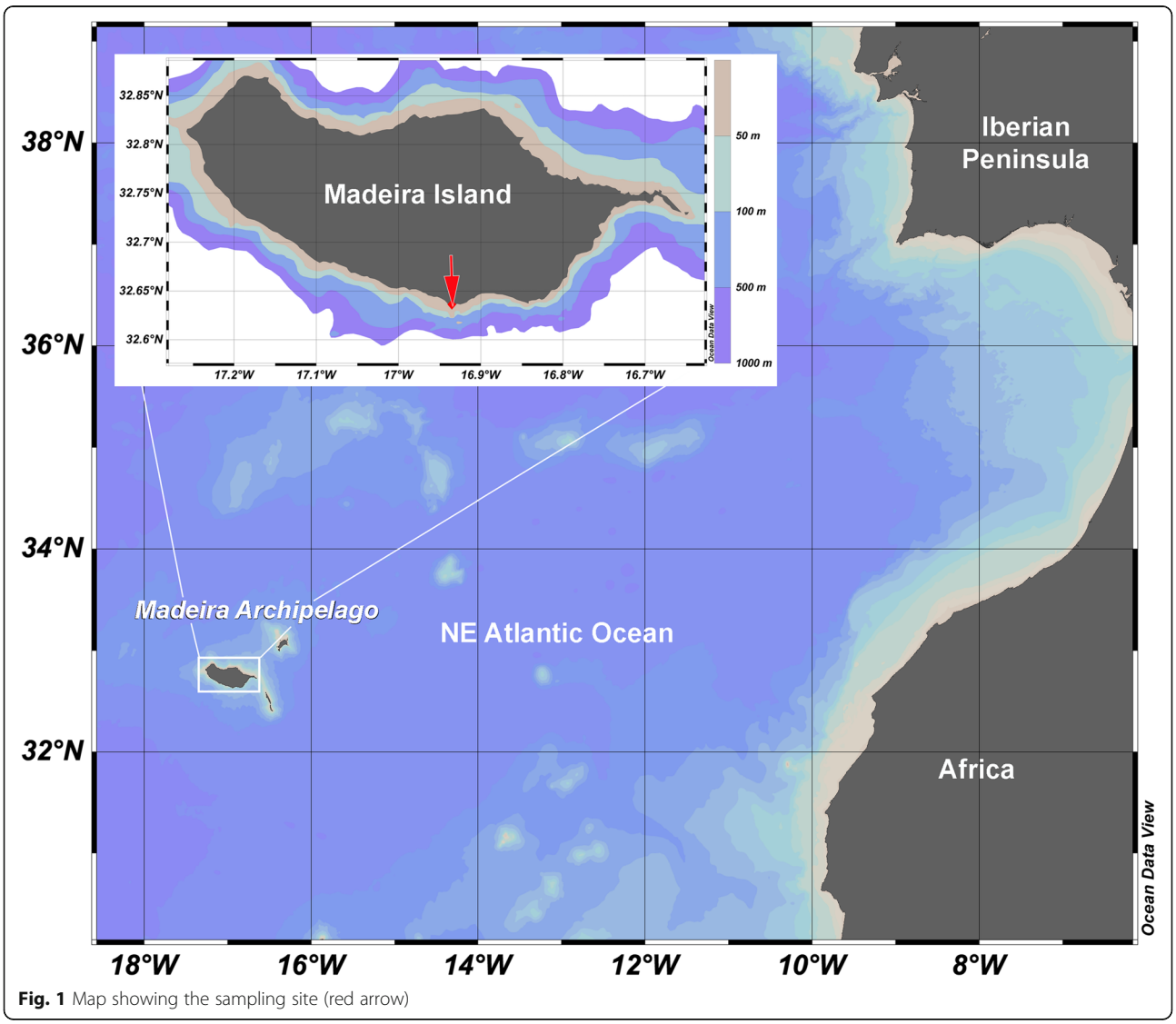

Fig. 1 Map showing the sampling site (red arrow)

confirmed the occurrence of a species of *Gambierdiscus* from 2008 onwards in the Selvagens islands as well as on Madeira (Kaufmann et al., 2015) and the initial species identification, as *G. excentricus*, was provided but without morphological or genetic verification.

The aim of this study was to isolate *Gambierdiscus* from Madeira and to reliably identify the species through an integrative taxonomic approach, combining morphological and molecular data.

Methods

Macroalgae (*Halopteris* and *Padina*) was sampled on March 21 2014 by SCUBA-diving collecting several individual thalli carefully within a plastic bag with surrounding seawater at a depth of about 6–7 m in Cais do Carvão Bay, Funchal, south coast of Madeira (32° 38.170′N 16° 56.110′W, Fig. 1). This small bay is characterized by a gentle slope with rocky substrate, i.e. basaltic rocks covered with sessile biota of mainly brown and some red macroalgae species, sponges, sea anemones, sea urchins and sea cucumbers. At about 10–12 m depth the rocky substrate transitions to sandy substrate. The south coast of Madeira Island is protected from the prevailing NE-Trade winds, so the wave action is rather low with prevailing westerly waves of less than 2 m significant height and mean sea surface temperatures ranging from 18 °C (February/March) to 23–24 °C (August/September).

The sample was brought to the laboratory within less than 1 h after collection for further analysis. In the laboratory, the plastic bag was vigorously shaken for about a minute to dislodge benthic dinoflagellates from the seaweeds. The seaweed was withdrawn from the bag and subsamples of the remaining seawater containing the dislodged dinoflagellates were observed for identification and isolation of single cells.

Isolated cells were washed in fine-filtered seawater from the sampling site and transferred into a small Petri dish containing filtered seawater first and later small amounts of f/2-medium (Guillard & Ryther, 1962) were added. After several weeks a slowly growing unialgal culture was established. Unfortunately, the culture died shortly after our first preparation of cells for light and scanning electron microscopy.

Living cells or an empty theca of interest were picked using a Leica DMIL inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany), placed on an object slide and observed with a Leica DMRB (Leica Microsystems GmbH, Wetzlar, Germany) equipped with differential interference contrast optics at 400 times magnification. Digital photos were taken using Leica DFC290 and DFC420C cameras (Leica Microsystems GmbH, Wetzlar, Germany).

For scanning electron microscopy (SEM), living cells were fixed with Lugol solution and empty (dead) cells were used directly. Cells were placed on a 5 μm Millipore filter, rinsed with distilled water several times, air dried at room temperature and finally at 50 °C in a drying oven for 5 mins. The filter was mounted on a stub and sputter coated with gold-palladium (Bal-Tec SCD 050; BAL-TEC Präparations-Gerätevertrieb, Wallof, Germany). Cells were observed using a Tescan VEGA3 microscope (Elektronen-Optik-Service GmbH, Dortmund, Germany) at 15 kV using the secondary electron (SE) and the back scatter electron (BSE) detectors. SEM images were presented on a black background using Adobe Photoshop CS6.

Labelling of epithecal plates follows the traditional Kofoid system of plate series (Litaker et al., 2009; Hoppenrath et al., 2014; opposed to Fraga et al., 2011) with disregard of possible plate homologies. The hypothecal interpretation is in light of gonyaulacalean relationships (two asymmetric antapical plates of different size and no posterior intercalary plate) with a posterior sulcal plate outside the depressed longitudinal furrow (Fraga et al., 2011; Hoppenrath et al., 2014; opposed to Litaker et al., 2009).

Genomic DNA was extracted from 20 living cultured cells using the MasterPure Complete DNA and RNA Purification Kit (EPICENTRE, Madison, WI, USA). The final DNA pellet was dried and sent to the University of Technology Sydney, Australia.

The pellet was used as a template for large subunit (LSU) and small subunit (SSU) ribosomal DNA sequence amplification in 25 μL reactions in PCR tubes. Final concentration of reactions consisted of 0.6 μM forward and reverse primer, 0.4 μM BSA, 2–20 ng DNA, 12.5 μL 2xEconoTaq (Lucigen Corporation, Middleton,WI, USA), and 7.5 μL PCR grade water.

The PCR cycled initially for 10 min step at 94 °C, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, finalized with 3 min of extension at 72 °C. The LSU D8-D10 and SSU rDNA regions were amplified with the FD8-RB and 18ScomF1-18ScomR1 primer sets, respectively. The LSU amplicon was sequenced with the FD8-RB primer set, the SSU amplicon was sequenced with the 18ScomF1-Dino18SR1, G10'F-G18'R and Dino18SF2-18ScomR1 primer set combination (Kretzschmar et al., 2017). Sanger sequencing was conducted by Macrogen Inc. (Seoul, Korea).

Sequencing results were aligned with *Gambierdiscus* spp. data from the GenBank reference database (accession numbers as part of Figs. 4 and 5). Alignment, phylogenetic inferences and visualization were conducted within the Geneious software, version 10.1.7 (Kearse et al., 2012). Alignments with a maximum of eight iterations using the algorithm MUSCLE (Edgar, 2004) were truncated to uniform length (D10-D8 LSU

and SSU at 787 and 1708 bp, respectively) and discrepancies removed. Bayesian inference (BI) and maximum likelihood (ML) methods were used to infer phylogenetic trees. Posterior probability (PP) distribution was estimated with Mr. Bayes 3.2.2 for BI with Metropolis-Coupled Markov Chain Monte Carlo simulations (Ronquist & Huelsenbeck, 2003). Three heated and one cold chain(s) with random starting tree was used with a temperature set at 0.2. Trees were sampled every 100th generation for the 2,000,000 generations generated. The ML analysis used PHYML with 1,000 bootstraps (BS) (Guindon & Gascuel, 2003). For both BI and ML analyses the general time reversal model with an estimated gamma distribution was used. Branch support was analyzed as follows (PP/BS respectively): 1.00/100% was fully supported, well supported constituted above 0.9/90%, above 0.8/80% was relatively well supported and unsupported if below 0.5/50%.

Results

Morphological observations

Cells had brown chloroplasts (Fig. 2a) and were 95 to 105 μm deep and 100 to 110 μm wide (Fig. 2a-e). The observed tabulation was 3′ 7′′ ?C 4+S 5′′′ 2′′′′ (Fig. 3a-i). Cells were lens-shaped and anterio-posteriorly compressed. Thecal plates were smooth with many scattered pores (Fig. 3a-c, h). The pore plate (Po) was ventrally displaced (Figs. 2, 3c-e, a, b) and had a fish-hook shaped pore that showed some variability in its shape (Fig. 3d-g). The normal pores on the Po plate were irregularly distributed (Fig. 3d-g). The *2′′′′* plate was not in contact with plate *5′′′* and about one third of the cells transdiameter wide (Fig. 2b) but sometimes wider (Fig. 3c).

Molecular phylogenetic analyses

The D8-D10 LSU rDNA for both BI and ML resolved the same topology (Fig. 4). *Gambierdiscus* spp. clustered in the previously established pattern for the D8-D10 LSU rDNA gene tree (e.g. Kretzschmar et al., 2017). The Madeira isolate resolved among other *G. excentricus* isolates with full support for both ML and BI analyses (Fig. 4). The genetic distance between *G. excentricus* isolates was comparatively small, ~ 0.5%. The closest sister clade was *G. australes* with genetic distances ~ 9%.

The SSU rDNA topology resolved the major *Gambierdiscus* clades with full or high support, with the exception of the deep branch separating the *G. carolinianus*, *G. polynesiensis* and *G.* sp. 3 clade with the *G. excentricus* and *G. australes* clade (Fig. 5). The isolate from Madeira Island was sister to the *G. australes* clade (Fig. 5).

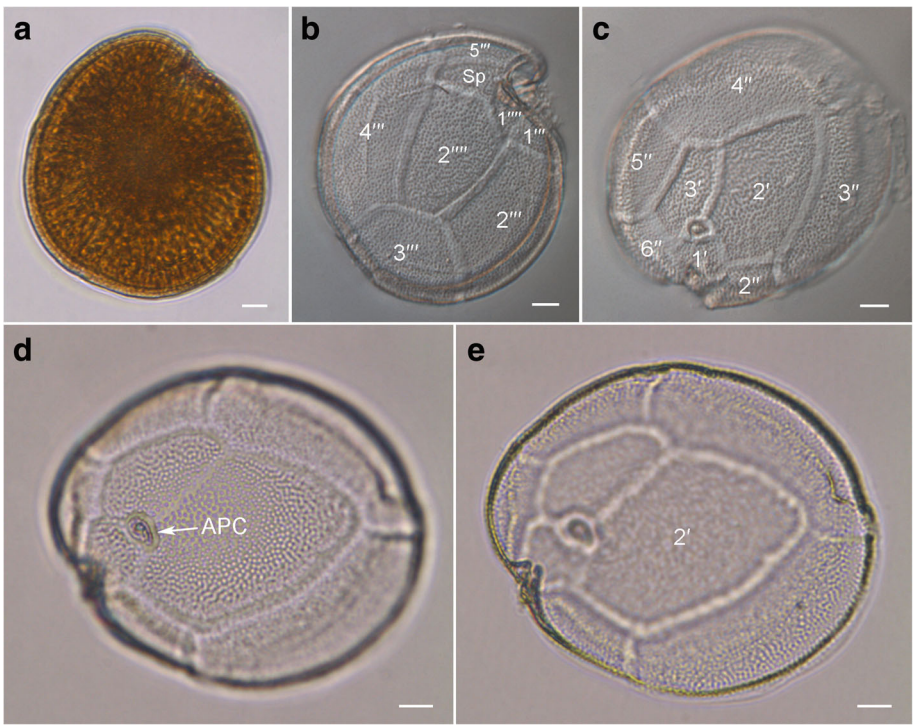

Fig. 2 Light micrographs of *Gambierdiscus excentricus*. **a**: Bright field (BF) micrograph showing a cell in mid cell focus. **b**: DIC micrograph of an empty hypotheca, note the tabulation. **c**: DIC micrograph of an empty epitheca, note the tabulation. **d**, **e**: BF micrographs of an empty epitheca in different focal planes showing the tabulation. Scale bars = 10 μm

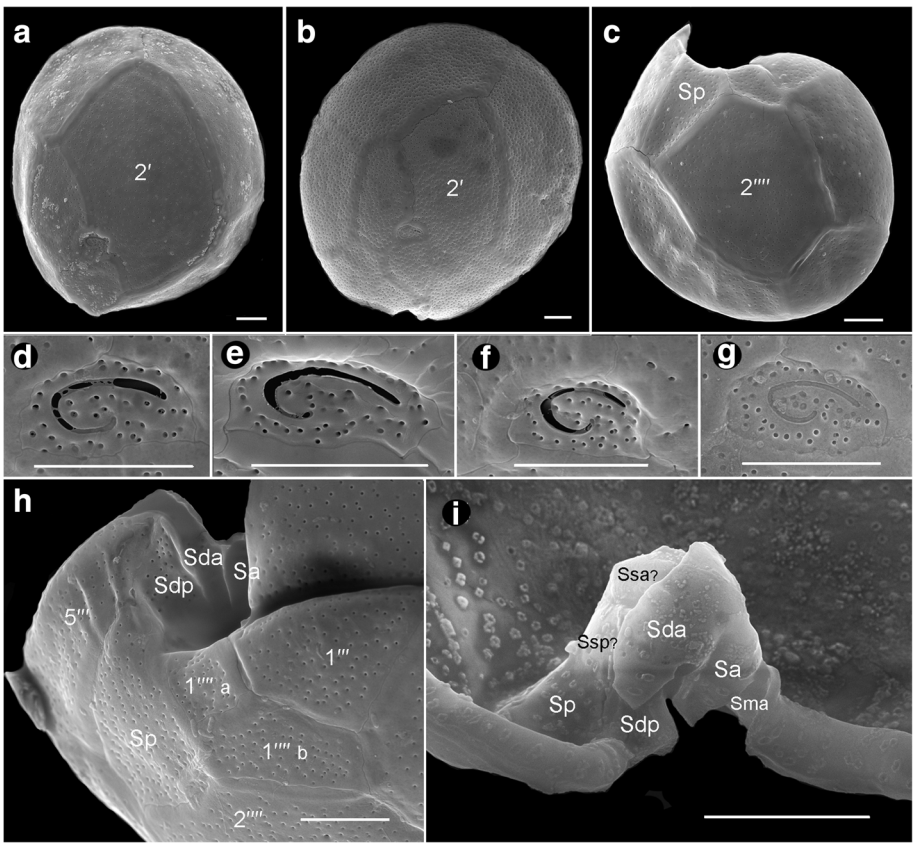

Fig. 3 Scanning electron micrographs of *Gambierdiscus excentricus*. **a**, **b**: Apical view of the epitheca showing the tabulation. **c:** Antapical view of the hypotheca showing the tabulation. **d-g**: Details of the APC microarchitecture. Note the different shapes of the hook-shaped apical pore and the different arrangements of the pores in the pore plate. **h**: Ventral view of the hypotheca showing part of the sulcus. The first antapical plate is split in two parts (aberrant cell). **i**: Inside view on the anterior part of the sulcal pocket. Scale bars = 10 μm

The genetic distance between the Madeira isolate and *G. australes* was ~ 13.5%.

Discussion

The first record of a species of *Gambierdiscus* in the Madeira archipelago was reported from a sample fixed in formaldehyde taken in 2008, and was verified by a live sample in 2012 (Kaufmann et al., 2015). An initial species identification as *G. excentricus*, was provided in a phytoplankton checklist but without morphological or genetic verification (Kaufmann et al., 2015). The present study clearly identifies the presence of *G. excentricus* at the Madeira archipelago based on morphological features as documented using light and scanning electron microscopy, and a phylogenetic analysis using regions of the ribosomal RNA genes. The observed morphology generally conformed to the original description of *G. excentricus* (Fraga et al., 2011) (Table 1). Variability in the shape of the apical pore and the microarchitecture of the apical pore plate were documented for the first time as well as variability in the width of the second antapical plate. The APC variability is known already for other *Gambierdiscus* species and therefore its micromorphology has not been used as species character (Litaker et al., 2009). The width of the 2′′′ plate alone is no specific feature. It has been used only in combination with epithecal plate patterns. So that variability will cause no problems to identify the species.

The LSU rDNA sequence clustered together with other *G. excentricus* isolates with high support values (Fig. 4), supporting the close relationship between this isolate and those previously cultured. The first SSU rDNA sequence for the species *G. excentricus* is reported in this study. It clustered as a sister taxon to the *G. australes* clade (Fig. 5) and thus supported the close relationship of both species. Contrary to the results based on our own (Fig. 4) and previously published phylogenies of the LSU rDNA regions (Fraga et al., 2011; Nishimura et al., 2013), the *G. carpenteri* and *G. caribaeus* clade was not the sister clade to this group, instead a *G. carolinianus* and *G. polynesiensis* clade branched as sister group (Fig. 4).

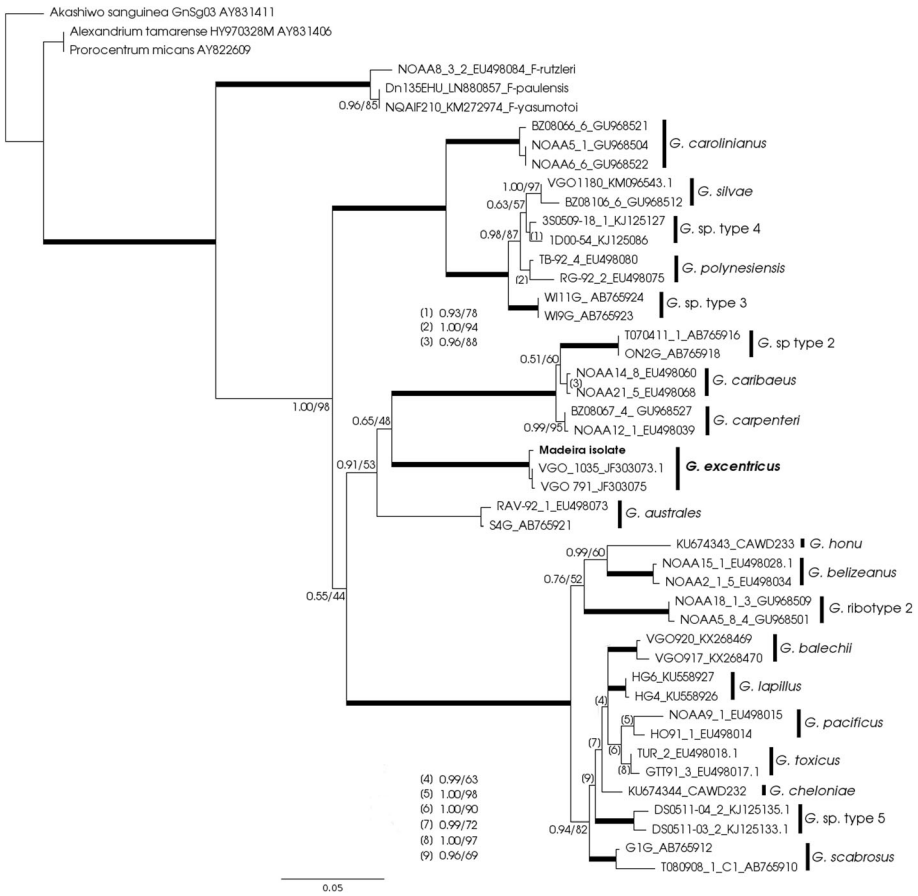

Fig. 4 Maximum likelihood phylogeny of *Gambierdiscus* species/phylotypes of the D8-D10 LSU rDNA region. Nodal support is Bayesian posterior probability (PP) and maximum likelihood bootstrap (BS) values obtained from Bayesian inference analysis and maximum likelihood analysis, respectively. Nodes with strong support (PP/BS = 1.00/100) are shown as thick lines

This discrepancy of deeper clade resolution between different rDNA gene regions has been previously observed in *Gambierdiscus* (e.g. Kretzschmar et al., 2017; Nishimura et al., 2014; Nishimura et al., 2013) and is likely due to the difference in SSU and LSU rDNA evolutionary rates (Murray et al., 2005) or different gene evolutionary histories to the species evolution (e.g. Degnan & Rosenberg, 2006). In both phylogenetic analyses for both rDNA gene trees the statistical support for the deep branching order for the clade containing *G. excentricus* and *G. australes* was varied from relatively supported to unsupported, so that the closest relatives of *G. excentricus* and *G. australes* in the species evolution are unresolved. In general, our phylogenetic trees supported a similar branching pattern within *Gambierdiscus* as found in previous studies, including the existence of sub-clades, comprising a large clade containing *G. scabrosus, G. toxicus, G. cheloniae, G. pacificus, G. lapillus, G. balechi, G. belizeanus, G. honu*; a clade of *G. carpenteri, G.* sp type 2, and *G. caribaeus*; and a clade of *G.polynesiensis, G. carolinianus, G. silvae* and *G.* sp type 3 (Nishimura et al., 2014; Fraga & Rodriguez, 2014; Smith et al., 2016; Kretzschmar et al., 2017; Rhodes et al., 2017; Nishimura et al., 2013).

Regions of SSU rDNA, in particular, the V4 and V9, have been extensively used as barcoding markers for marine microbial eukaryotic species present in environmental DNA (ie De Vargas et al., 2017), including for benthic dinoflagellates (Smith et al., 2017). The *G. excentricus* V4 and V9 SSU sequences obtained for the first time in this study will allow for the evaluation of these sequences for their use in future barcoding studies.

It is not yet clear which species of *Gambierdiscus* are responsible for producing the CTX congeners which can result in CFP. Using LC-MS/MS techniques, which can identify specific congeners of toxins, known CTX congeners have so far only been found in cultures of the species *G. polynesiensis* (Chinain et al., 2010; Rhodes et al., 2014). *Gambierdiscus polynesiensis* was first described from French Polynesia in the Pacific Ocean region (Chinain et al.,

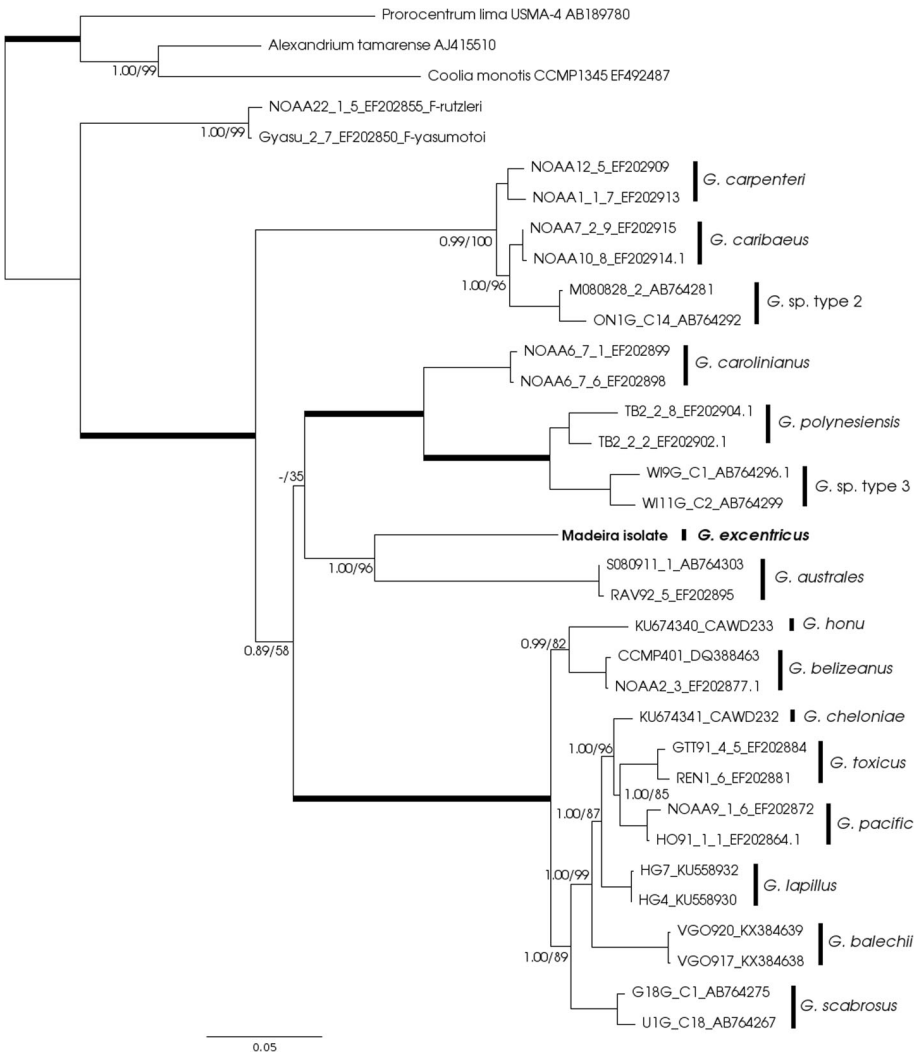

Fig. 5 Maximum likelihood phylogeny of *Gambierdiscus* species/phylotypes of the SSU rDNA region. Nodal support is Bayesian posterior probability (PP) and maximum likelihood bootstrap (BS) values obtained from Bayesian inference analysis and maximum likelihood analysis, respectively. Nodes with strong support (PP/BS = 1.00/100) are shown as thick lines

1999), and produces both Type 1 (CTX-4A, CTX-4B) and Type 2 (CTX-3C, M-seco-CTX-3C, 49-epiCTX-3C) P-CTXs in culture (Chinain et al., 2010; Rhodes et al., 2014). While other species have been found to produce abundant MTX-1 and MTX-3 using LC-MS (Rhodes et al., 2014), no other species of the species tested has so far shown identifiable CTX production with this method.

In the species *G. excentricus*, CTX toxicity using LC-MS/MS has not yet been tested. However, using a cell-based assay method, the Neuro-2a cell based assay (NCBA) (Fraga et al., 2011; Pisapia et al., 2017; Litaker et al., 2017), which analyses the mode of action of particular toxin fractions, it appears that *G. excentricus* produces CTXs, and appears to be more toxic than comparable *Gambierdiscus* species. The *G. excentricus* strain tested in the (Litaker et al., 2017) study was ~ 44- to 1,740-fold more toxic than the other examined species. *G. excentricus* is present and abundant in the eastern Atlantic (Canary Islands; Fraga et al., 2011; Rodríguez et al., 2017), and has been found in Brazil (Nascimento et al., 2015) and in the Caribbean Sea (Litaker et al., 2017). Therefore, we suggest that this species may be likely to be the causative species for the production of CTX toxins related to CFP poisoning cases in Madeira.

Conclusions

This is the first reliable species identification of *G. excentricus* at the Madeira archipelago based on morphological

Table 1 Morphological comparison of *Gambierdiscus excentricus* records

	Canary Islands[1]	Brazil[2]	Madeira[3]
Sampling site	28° 34.62′N	22° 45.3′S	32° 38.17′N
	16°19.7′W	41°54.12′W	16° 56.11′W
Cell shape	lenticular	lenticular	lenticular
Cell compression	anterio-posteriorly	anterio-posteriorly	anterio-posteriorly
Cell size			
depth [μm]	84–115	60–95	95–105
width [μm]	69–110	53–91	100–110
thecal ornamentation	smooth with pores	smooth with pores	smooth with pores
plate formula	Po 3′ 7″ 6c ?s 5‴ 2⁗	Po 3′ 7″ 6c 8s? 5‴ 2⁗	Po 3′ 7″ ?c 4+s 5‴ 2⁗
APC			
location	ventrally displaced	ventrally displaced	ventrally displaced
apical pore	fishhook-shaped slit	fishhook-shaped slit	fishhook-shaped slit
marginal pores	one row	one row	irregular
Plate 2′	~ rectangular	~ rectangular	~ rectangular
Ratio 2′/3′ to 2′/1′	~ 2	2.2–2.6	~ 2
Plate 2⁗	narrow	narrow	narrow to wide

[1](Fraga et al., 2011), [2](Nascimento et al., 2015), [3]present study

and a phylogenetic analysis using regions of the ribosomal RNA genes. The first SSU rDNA sequence for the species *G. excentricus* was gained. This record suggests that *G. excentricus* may be the causative species for the production of CTX toxins related to CFP poisoning cases in Madeira, a hypothesis that needs verification in future.

Abbreviations

APC: apical pore complex; BI: Bayesian inference; BS: Bootstrap support; BSA: Bovine serum albumin; BSE: Back scatter electron; CFP: Ciguatera fish poisoning; CTXs: Ciguatoxins; DNA: Deoxyribonucleic acid; LC-MS/MS: Liquid chromatography–mass spectrometry/mass spectrometry; LSU rDNA: Large subunit ribosomal DNA; ML: Maximum likelihood; MTXs: Maitotoxins; PCR: Polymerase chain reaction; Po: outer pore plate; PP: Posterior probability; SE: Secondary electron; SEM: Scanning electron microscopy; SSU rDNA: Small subunit ribosomal DNA

Acknowledgements

We would like to thank M. Böhm-Beck, Marine Biology Station of Funchal, for support in the laboratory and J. Ortgies, Senckenberg am Meer, for technical assistance. SM and LK thank the Australian Research Council FT scheme for funding. This is a contribution to project INTERREG MAC 2014-2020 MIMAR MAC/4.6d/066. This is contribution number 45 of the Funchal Marine Biology Station.

Authors' contributions

MH: cell isolations, culturing, light and scanning electron microscopy, DNA extraction, drafting and editing the manuscript; ALK: PCR, phylogenetic analyses, editing the manuscript; SAM: infrastructure and salary support, editing the manuscript; MJK: sampling, infrastructure, editing the manuscript. All authors read and approved the final manuscript.

Funding

This is a contribution to project INTERREG MAC 2014–2020 MIMAR MAC/4.6d/066. SM was supported by an Australian Research Council Future Fellowship grant FT120100704, and ALK was supported by an UTS Doctoral scholarship.

Availability of data and materials

The data and datasets used and analysed during the current study are available from the first and corresponding authors on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 23 March 2018 Accepted: 19 July 2019
Published online: 14 August 2019

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