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Gambierdiscus balechii sp. nov (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean)

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ABSTRACT

A new benthic toxic dinoflagellate is described from the Celebes Sea. *Gambierdiscus balechii* sp. nov. was isolated from seaweeds growing in tidal ponds. Its morphology was studied by means of LM and SEM; *G. balechii* has a very ornamented theca, a hatchet shaped second apical plate, a narrow second antapical plate and an asymmetrical third precigular plate, a unique combination of characters among *Gambierdiscus* species. It has a very wide size range with widths from 36 to 88 μm. Phylogenetic analyses of two *G. balechii* strains, based on LSU rRNA (D8–D10) and partial SSUrRNA sequences confirmed that these clustererd in its' own group, separated from the rest of *Gambierdiscus* species and with *G. pacificus*, *G. belizeanus* and *G. scabrosus* as its closest relatives. Thecate cysts are described from culture as non motile vegetative-like cells which germinated after being isolated and transferred to fresh medium. Mouse tests showed that this species is toxic and hence it is a potential cause of ciguatera in the Celebes Sea.

1. Introduction

Benthic dinoflagellates have recently gained great interest due to the increasing incidence of their harmful effects (GEOHAB, 2012; Lemée et al., 2012; Parsons et al., 2012). The most important of these harmful effects is ciguatera fish poisoning (CFP) which is the most common type of marine food poisoning worldwide (De Fouw et al., 1999) affecting mainly tropical and sub-tropical marine areas like Caribbean Sea, Polynesia and other areas in the Pacific Ocean and in the Indian Ocean (Friedman et al., 2008; Lewis, 2006), although it is uncommon in Indonesia (Lehane and Lewis, 2000). CFP is caused by ciguatoxins (CTXs) produced by benthic dinoflagellates of genus *Gambierdiscus* (Yasumoto et al., 1977), although the role in this syndrome of additional toxins produced by other dinoflagellates cannot be discarded. The same genus may also produce other toxins i.e. gambieric acid (Nagai et al., 1992), gambierol (Satake et al., 1993a) and maitotoxins (MTXs) (Satake et al., 1995). MTXs are very potent toxins and have been found in the viscera of herbivorous fish but are unlikely to produce human illness due to their low capacity for bioaccumulation in fish tissue and low oral potency. The first report of *Gambierdiscus*, but under the name of *Goniodoma* sp., was from Cabo Verde islands, in the NE Atlantic Ocean, which was observed as an abundant species in October 1948 (Silva, 1956) but it was not related to any toxic event. *Gambierdiscus toxicus* Adachi et Fukuyo (Adachi and Fukuyo, 1979) was considered the only species of the genus until 1995 when the second species, *G. belizeanus* was described (Faust, 1995). Today, genus *Gambierdiscus* has now up to eleven described species: *G. pacificus* Chinain et Faust, *G. australes* Faust et Chinain, *G. polynesiensis* Chinain et Faust (Chinain et al., 1999), *G. caribaeus* Vandersea, Litaker, Faust, Kibler, Holland & Tester, *G. carolinianus* Litaker, Vandersea, Faust, Kibler, Holland & Tester, *G. carpenteri* Kibler, Litaker, Faust, Holland, Vandersea, & Tester (Litaker et al., 2009), *G. excentricus* S. Fraga (Fraga et al., 2011), *G. scabrosus* T. Nishim., Shin. Sato & M. Adachi (Nishimura et al., 2014), and *G. silvae* S. Fraga & F. Rodríguez (Fraga and Rodríguez, 2014). Recently, two former *Gambierdiscus* species, *G. ruetzleri* Faust, Litaker,

Abbreviations: CTX, ciguatera; GTA, glutaraldehide; CLSM, confocal laser scanning microscopy.

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Vandersea, Kibler, Holland & Testerand, and *G. yasumotoi* M.J. Holmes (Holmes, 1998; Litaker et al., 2009) which displayed a globular shape instead of the typical lenticular form, were transferred to the new genus *Fukuyoa* Gomez, Qiu, Lopes & Lin (Gomez et al., 2015), together with a third species, *F. paulensis* Gomez, Qiu, Lopes & Lin. Nonetheless, phylogenetic studies on natural samples have revealed a number of molecular clades in genus *Gambierdiscus*, not yet accepted as taxonomical entities, suggesting a broader specific diversity in this genus than presently recognized (Litaker et al., 2010; Nishimura et al., 2013; Nishimura et al., 2014; Xu et al., 2014).

The morphological differences among different species are sometimes very subtle making very difficult, or even impossible if few cells are available, the proper identification without the aid of molecular tools (Litaker et al., 2009). Although some species groups can be made based on the shapes and proportions of some plates like 2′, 3″ and 2⁗, the variability is very high. The differences among the two cells obtained after cell division was already reported for *G. excentricus* (Fraga et al., 2011) and can be considered as a general rule for all *Gambierdiscus* species. Differences on the cell length and relative size of plates was also reported for *G. silvae* were tall cells with big precingular plates were reported (Fraga and Rodríguez, 2014). Bravo et al. (2014) described the wide range in cellular size and the cellular and nuclear morphological variability of a single strain of *Gambierdiscus* which in this paper is described *Gambierdiscus balechii* sp. nov, a new toxic dinoflagellate found in the Indonesian coast at the Southern Celebes Sea. The characterization of *G. balechii* has been established on the basis of its distinct morphological features and molecular phylogeny.

2. Materials and methods

2.1. Source of specimens and culture conditions

Samples were collected in the coast of Manado, Indonesia (1°29′ N, 124°50′E) (Fig. 1) on February 17, 2007. Samples of *Ectocarpus* sp. were taken from a tidal pond, placed in plastic bottles and shaken. Afterwards, the gross particles were removed and the remaining seawater was used for cell isolation. Samples were sent to the IEO laboratory in Vigo, Spain, where cells of *Gambierdiscus* were isolated by a capillary pipette with the aid of a Zeiss Invertoscop D microscope (Carl Zeiss, Jena, Germany). Isolated cells were incubated in 96 microwell plates in half strength K medium without silicates (Keller et al., 1987) made with seawater from Ría de Vigo (NW Spain) with a salinity adjusted to 34 and incubated at 25 °C and a photon irradiance of about 90 μmol m^{-2} s^{-1} of PAR measured with a QSL-100 irradiameter (Biospherical Instruments Inc., San Diego, CA, USA) and at a 12:12 L:D photoperiod. The cultures were transferred to higher volumes for routine maintenance. Two cultured strains were obtained, VGO917 and VGO920, and both are maintained at the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo.

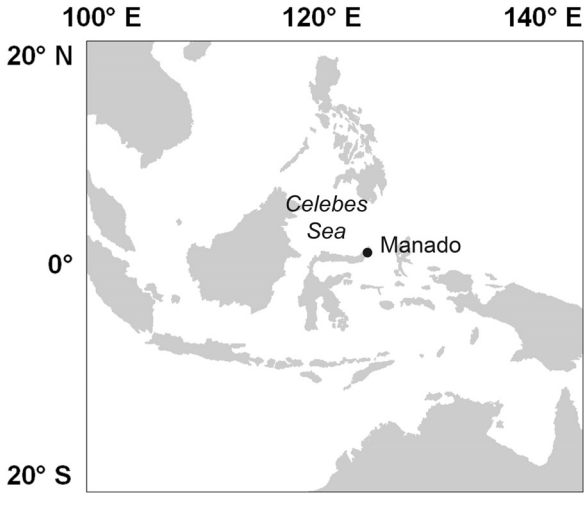

Fig. 1. Map of the Celebes Sea showing the locality where *Gambierdiscus balechii* was found.

2.2. Cyst production in cultures

Cyst production was induced in intercrosses and intracrosses of strains VGO917 and VGO920, using L1 and K/2 media (Guillard and Hargraves, 1993; Keller et al., 1987) without addition of N and P sources following the methodology described by Figueroa and Bravo (2005). The crosses were cultured under the same conditions described for culture maintenance and monitored every 2–3 days in a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Jena, Germany). Non motile cells considered as putative cysts were isolated with micropipette after one and a half months after crossing and transferred to 96 wells tissue culture plates with 0.2 mL of a mixture of K/2 and L1 (1:1) media in each well in order to provide conditions favorable to germination. Moreover, non motile cells were isolated from cultures in stationary phase of strains VGO917 and VGO920 growing in replete medium (K2 + L1, 1:1). Thirty cysts in total were isolated after being previously photographed using a Canon EOS-D60 digital camera.

2.3. Light microscopy

Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast, differential interference contrast and epifluorescence with an UV lamp and with UV and blue excitation filters. The cultured cells were observed alive or fixed with formalin. For plate pattern identification the cells were stained with Fluorescent Brightener 28 (Sigma–Aldrich, St Louis, MO, USA) following a modified technique (Fritz and Triemer, 1985). Other cells were dissected, squashing the cells by gently pressing the cover slip over them occasionally with the aid of sodium hypochlorite. Microphotographs were taken with a Canon EOS-D60 (Canon Inc., Tokyo, Japan) digital camera or with an Axiocam HRc (Carl Zeiss, Jena, Germany). When the depth of field was not enough for the whole object, several pictures were taken at a series of different foci and were automatically merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).

2.4. Scanning electron microscopy

Two methods have been used for SEM. Five mL of exponentially growing cultures were fixed with GTA at a final concentration of 4%. After 2 h at room temperature, they were rinsed three times with distilled water and dehydrated in a series of 30, 50, 75, 95 and 100% EtOH and 100% HMDS. In order to obtain images with a theca without the outer layers of the amphiesmal vesicles to expose the cellulose plates, 5 mL of culture were fixed with ethanol at a final concentration of 25% for 45 min and rinsed with distilled water. The filters were covered by a drop of 2% OsO_2 for 30 min and then dehydrated in a series 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 Phillips XL30 or a FEI Quanta 200 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

2.5. Size measurements

Width and depth were obtained from Bravo et al. (2014) who measured 1315 cells of strain VGO917 under Leica DMLA (Leica DMR, Germany) light microscope with an Axiocam HRc digital camera (Zeiss, Jena, Germany). Measurements of length were done based on SEM images as these measurements are difficult to obtain under a light microscope due to the anterioposterior compression of the cells. The width and length of 13 cells was determined to obtain the length/width ratio.

2.6. Nomenclature

In this study, a modified Kofoid tabulation system (Kofoid, 1909) as described in Besada et al. (1982) and used in the descriptions of *G. excentricus* (Fraga et al., 2011), *G. scabrosus* (Nishimura et al., 2014) and *G. silvae* (Fraga and Rodríguez, 2014), was followed to name the plates therefore allowing comparisons with other genera of Goniodomaceae. The terms "length" as apical/antapical distance, "width" as transdiameter and "depth" as dorso/ventral distance were used for the cell dimensions.

2.7. DNA extraction

Exponentially growing cultures of *Gambierdiscus balechii* strains VGO917 and VGO920 (1.5 mL) were harvested by centrifugation (13,000 rpm, 5 min), the cellular pellets were rinsed in 1 mL distilled water, centrifuged again and the supernatant discarded. DNA extraction was done using a modified Chelex procedure (Fraga and Rodríguez, 2014; Richlen and Barber, 2005). Genomic DNA was quantified and checked for its purity in a Nanodrop Lite spectrophotometer (ThermoScientific, Waltham, MA, USA), and stored at −20 °C until further processing.

2.8. PCR amplification and DNA sequencing

The divergent domain D8–D10 of the LSUrRNA gene and partial SSUrRNA gene sequences were amplified using the pairs of primers FD8/RB and EUK-A/EUK-B and SR4-F/SR9-R, respectively (Chinain et al., 1999; Han et al., 2016; Medlin et al., 1988). Amplification reaction mixtures (25 μL) contained 1.25 mM $MgCl_2$, 0.25 pmol of each primer, 2.4 mM of dNTPs, 0.25 units Taq DNA polymerase (New England Biolabs, MA, USA), and 1–2 μL from the Chelex extractions. The DNA was amplified in a Surecycler 8800 thermocycler as follows: 4 min denaturing at 94 °C, followed by 30 cycles of 30 s denaturing at 94 °C, 1 min annealing at 54 °C and 2 min elongation at 72 °C, with an elongation step of 10 min at 72 °C. A 10 μL aliquot of each PCR reaction was checked by agarose gel electrophoresis (1.5% TAE, 80 V) and GelRed™ nucleic acid gel staining (Biotium, Hayward, CA, USA). The PCR products were purified with ExoSAP-IT (USB Corporation, OH, USA) and amplicons sequenced in both directions using the Big Dye Terminator v3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and migrated in an AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidade de Vigo, Spain) and the LightRun™ sequencing service (GATC Biotech AG, Germany). The amplified D8–D10 LSUrRNA and partial SSUrRNA gene sequences obtained in this study were deposited in GenBank (for Acc. Nos. see Figs. 9 and 10).

2.9. Phylogenetic analyses

Gambierdiscus sequences were aligned using CLUSTALW multiple alignment in MEGA 6 (Tamura et al., 2013) and both amplicons examined by eye to correct the final sequences. Net average genetic distances ($dA = dXY - (dX + dY)/2$, where dXY is the average distance between groups X and Y, and dX and dY are the mean within-group distances (Nei, 1987), were calculated between *Gambierdiscus* clades for the original alignments using MEGA 6 software. It must be remembered that an important assumption behind the Nei genetic distance method is that the divergence rates among species are equivalent. D8–10 LSUrRNA and SSUrRNA alignments included 906 and 734 positions, respectively. Phylogenetic model selection (ML) was performed on MEGA 6 and a K2 + G model was selected in both LSU and SSU phylogenies with gamma shape parameter ($\gamma = 0.215$ and $\gamma = 0.448$, respectively) and transition/transvertion ratios of 1.8376 and 4.6352, in each case. Genus *Fukuyoa* sequences were used to root the trees. The phylogenetic relationships were also determined using Bayesian phylogenetic inference and in this case the substitution models were obtained by sampling across the entire general time reversible (GTR) model space following the procedure described in Mr. Bayes v3.2 manual. Bayesian trees were performed with Mr. Bayes v3.2 (Huelsenbeck and Ronquist, 2001) and the program parameters were statefreqpr = dirichlet (1,1,1,1), nst = mixed, rates = gamma. The phylogenetic analyses involved two parallel analyses, each with four chains. Starting trees for each chain were selected randomly using the default values for the Mr. Bayes program. The corresponding number of unique site patterns for LSU and SSU alignments were 339 and 568, respectively. The number of generations used in these analyses was 1,000,000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization ("burn-in" phase). All final split frequencies were <0.02. Maximum Likelihood phylogenetic analyses were conducted in MEGA 6. Bootstrap values were estimated from 1000 replicates. Overall topologies by ML and Bayesian inference method were very similar. The phylogenetic trees were represented using the ML method with bootstrap values and posterior probabilities from the Bayesian inference.

2.10. Toxin analysis

2.10.1. Extraction

Cells of *G. balechii* strain VGO 917 were grown for CTXs characterization in Erlenmeyer flasks at 26 ± 1 °C, salinity of 32, under a 12:12 h light:dark regime in K/2 medium. Culture volumes ranged between 200 and 750 mL. Cultures at early stationary phase were filtered through Whatman GF/C glass fiber filters to harvest cells. A Lugol-fixed aliquot was collected to determine cell concentration under the light microscope using a Sedgewick–Rafter chamber. The filters containing cells were extracted four times in MeOH (34 mL for a total biomass of 1.05×10^7 cells) under sonication and centrifugation at 5065 × *g* for 10 min at 10 °C. Twenty six mL of the extract were evaporated and then possible CTXs and MTXs were separated by solvent partition of the resulting residue using 5 mL of dichloromethane and 2× 2.5 mL of aqueous methanol (MeOH:H_2O 60:40) according to the procedure recommended by Satake et al. (1993b). During liquid–liquid partitions, CTXs and MTXs were respectively recovered in the dichloromethane and aqueous methanol phases. In order to avoid carryover of MTX-like compounds into the dichloromethane phase and vice versa, both phases were handled with extreme care. Thus, 2 mL were collected from the upper MeOH aqueous phase and also from the bottom of the dicholoromethane phase.

2.10.2. Mouse bioassay (MBA)

Healthy male Swiss mice NMRI weight 20 ± 1 g were used. The stock colony for the assay is managed following the Council Directive (EC 2007) on the approximation of laws, regulations and administrative provisions of the EU Member States regarding the protection of animals used for experimental and other purposes. Aliquots of 2 mL of the dichloromethane and aqueous methanol soluble fractions, as well

as blanks of each of the solvents, were dried under vacuum and re-suspended in aqueous Tween 60 1%. Mice were intraperitoneally injected with 1 mL of Tween solution. Two mice were employed for each and every sample and solvent controls, including Tween solution controls. After inoculation, mice were observed over 24 h with food and water provided ad libitum, paying attention to toxicity signs (Lewis and Endean, 1983) and time to death.

3. Results

3.1. *Gambierdiscus balechii* S. Fraga, F. Rodríguez et I. Bravo sp. nov.

Diagnosis: Typical cells of *G. balechii* are anterioposteriorly compressed with a length/width ratio of 0.65, an average depth 57.4 ± 0.3 (32–77) μm, and width 60.4 ± 0.4 (36–88) μm, but other more rounded forms can also be observed among the smallest cells. Thecal plate formula: Po, 4′, 0a, 6″, 6c,?s, 5‴, 0p, 2⁗. Thecal plates are ornamented with multiple evenly distributed depressions which most of them have a pore in the bottom. Apical pore plate Po is oval with a fishhook-shaped slit with pores in the center and surrounded by a line of pores, and it is centrally located on the epitheca. First apical plate, 1′ is very small and can be easily observed on apical view. Second apical Plate 2′ is the largest of the epitheca and has the suture 2′/3′ about as long as the suture 2′/4′. The ratio of sutures 2′/1″ to 2′/3″ ranges from 0.30 to 0.89, average 0.63 (n = 71) which corresponds to a hatchet-shaped plate. Plate 3′ is asymmetrical. In the hypotheca, Plate 2⁗ is small and narrow in comparison with the postcingular plates, 1⁗ and Sp. The flagellar area forms a conspicuous hollow from which two flagella emerge, the longitudinal one being perpendicularly projected. Cells are photosynthetic and the chloroplasts are round and numerous. In many cells the nucleus is U shaped and is located in the dorsal part of the cell with the points toward the ventral side of the cell but oval or even round nuclei can also be observed.

Holotype: Fig. 2 obtained from strain VGO917, barcoded in GenBank (GenBank ID: KX268470), and (GenBank ID: KX384638) and with preserved DNA at Centro Oceanográfico de Vigo (IEO). Strain VGO917 was obtained from a single cell isolated from a sample collected on February 17, 2007 as an epiphyte on the macroalgae *Ectocarpus* sp. on a tidal pond in Manado, Indonesia (Fig. 1). It is maintained at the Culture Collection of Harmful Microalgae of Centro Oceanográfico de Vigo (CCVIEO) and has been deposited at Banco Español de Algas (BEA) Spain, under the strain name BEA 1530B.

Etymology: It honors the late Prof. Enrique Balech for his outstanding contributions to the taxonomy of armored dinoflagellates.

Type locality: Manado, Indonesia (1°29′ N, 124°50′ E) (Fig. 1).

Distribution: *Gambierdiscus balechii* is only known from the type locality.

3.2. Morphology

Armored cells, anteroposteriorly compressed, with a length/width ratio of 0.65 (n = 13), an average depth 57.4 ± 0.3 (32–77) μm (n = 1315), width 60.4 ± 0.4 (36–88) μm (n = 1315). This very wide cell size range has been studied and described by Bravo et al. (2014) where, based on cellular size and nuclear shape, cell were grouped in four clusters that might correspond to different life cycle stages were observed. In apical or antapical view the cell is round and slightly indented in the ventral area showing a lobe in the right side of variable size (Figs. 2B, C, 3A, B, 4A, B and 7A). The theca is thick and very ornamented although the degree of ornamentation depends on the age of the plates (Figs, 2, 3 and 7A). In recently divided cells, the plates which were inherited from the mother cell are deeply ornamented of the type reticulate-foveate in the sense used for genus *Prorocentrum* by Hoppenrath et al. (2013) while in the new plates formed after division they are smooth (Fig. 4A and B). Where plates overlap with others, the ornamentation is smoother. The plates are covered by evenly distributed round pores at a concentration of about 30–50 pores per 100 μm^{-2} which lie in the bottom of most of the depressions (Fig. 4C and D). In the edges of the plates in contact to the cingulum, there is a line of marginal pores which marks the limit of a wing around the cingulum (Fig. 4D).

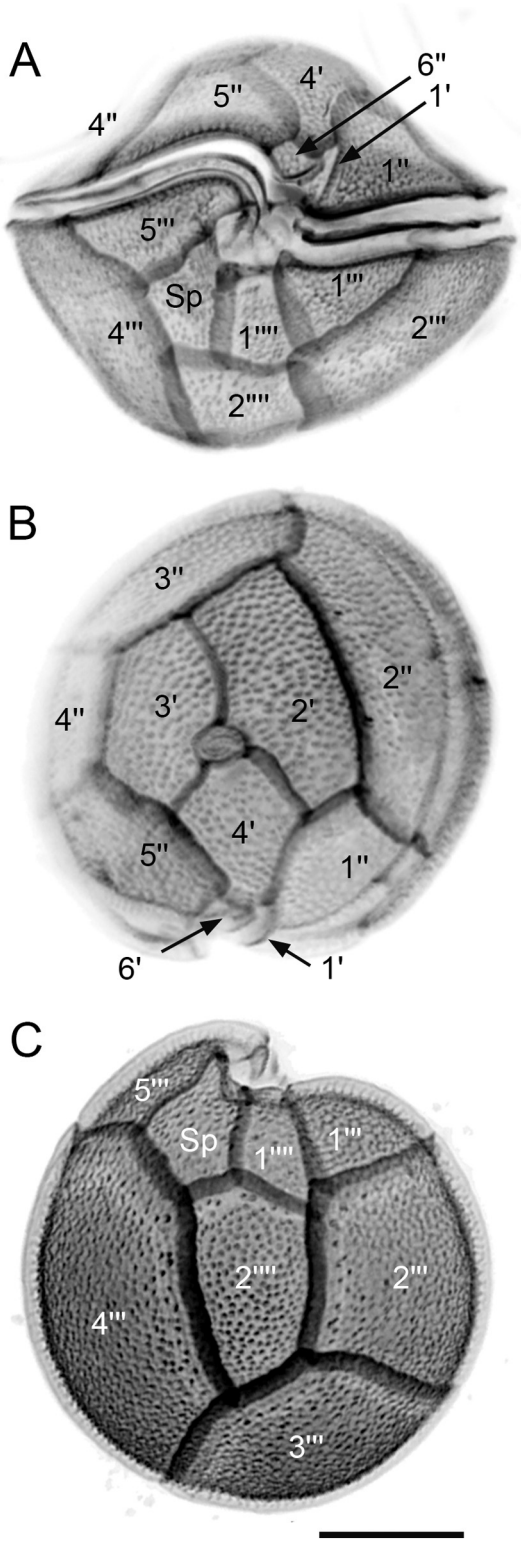

Fig. 2. *Gambierdiscus balechii* (Strain VGO917). CLSM images of calcofluor stained thecae. (A) Ventral view, (B) apical view, and (C) antapical view. Scale bar: 20 μm.

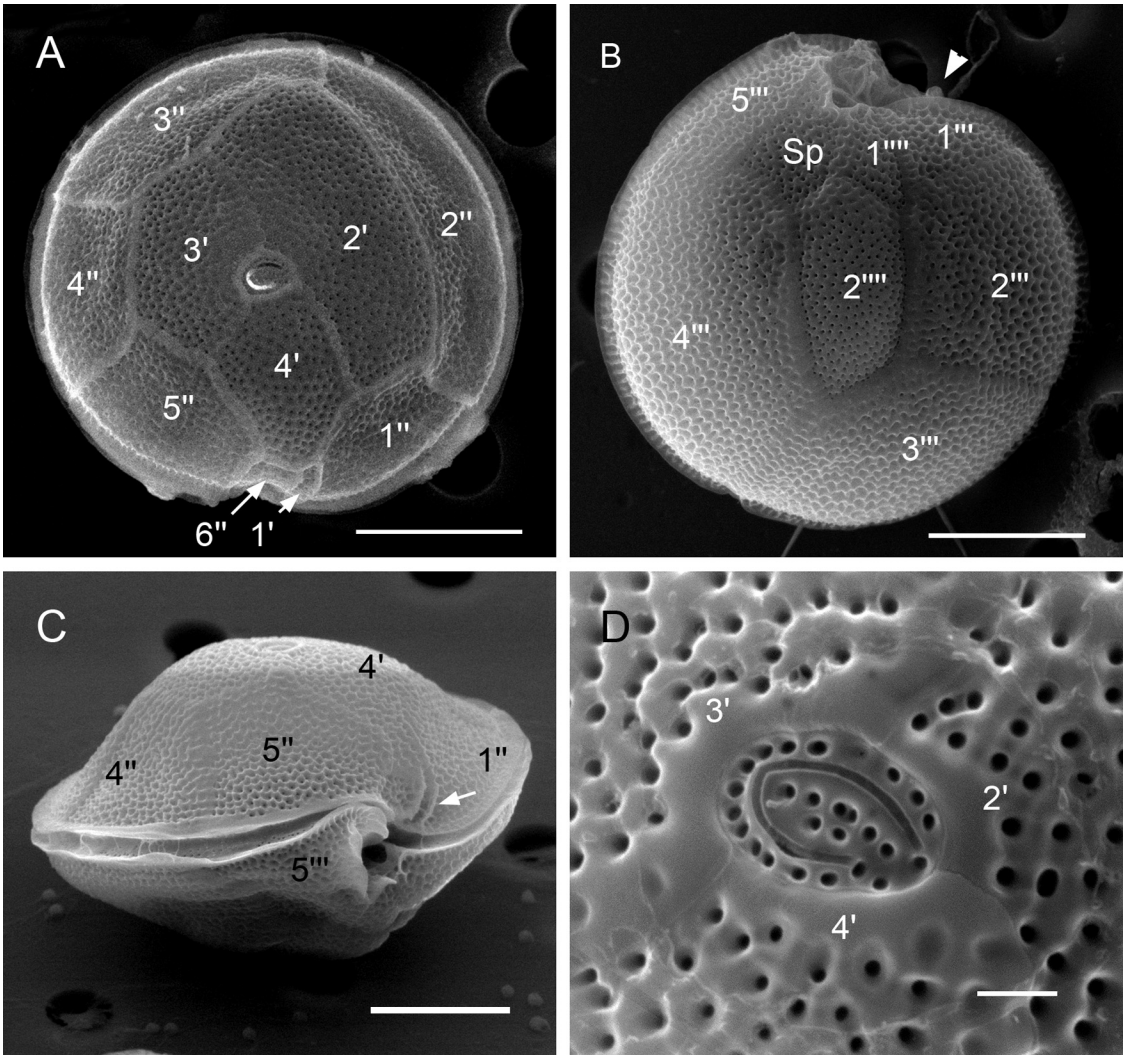

Fig. 3. SEM images of *Gambierdiscus balechii* (Strain VGO917). (A) Apical view, (B) antapical view. Arrowhead shows one keel-like edge of Plate 1′, (C) ventral view, (D) Po plate, scale bars: (A–C) 20 μm, (D) 2 μm.

The plate formula is Po, 4′, 0a, 6″, 6c,?s, 5‴, 0p, 2⁗. Epitheca is usually slightly larger than hypotheca (Figs. 2C and 3C) (Video S1 in the Supporting Information). Po is oval and situated almost centrally located in the epitheca, and has a fishhook-shaped slit surrounded by a row or pores (Fig. 3D). It contacts three apical plates: 2′, 3′ and 4′ which overlapped it (Fig. 3D). Plate 1′ is very small and narrow. It does not contact Po but contacts 4′ with its anterior right side, 1″ with the left side, and 6″ with the posterior right side. While the sutures of plates are generally smoothly overlapped, the sutures of 1′ with its neighbors form like a keel-like structure (Figs. 3B, 4B, 5A and B). Plate 2′ is the biggest of the apical series and hatchet-shaped: the sutures 2′/3′ and 2′/4′ are almost of the same size, and the ratio between the 2′/1″ to 2′/3″ suture lengths is 0.64 (s = 0.14, n = 72) (Fig. 6). Plates 3′ and 4′ are six sided, similar in size and almost symmetrical in shape (Figs. 2B and 3A). Plate 2″ is the largest of the precingular series followed by 3″. Plate 3″ is asymmetrical having suture 3″/2″ shorter than 3″/4″ (Figs. 3A and 4A). Plate 6″ is the smallest of the precingular plates and is five sided: left side contacts 1′, anterior side contacts 4′, right side contacts 5″, the posterior right side contacts c6 and the posterior left side, which is concave, contacts sulcal anterior plate (Sa) (Figs. 2A, 3A and 5B). Precingular and postcingular plates form like wings in their contact to the deep cingulum with a distinct ornamentation from the rest of the plates in form of radial ribs (Figs. 3B, 4D, 5A and B). As in all *Gambierdiscus*, the cingulum is descendent about one girdle width but, in ventral view the flagellar area appears twisted clockwise giving the appearance of being ascendant (Figs. 2A, 3C, 5A and B). It is composed of 6 plates being c1 and c6 curved due to the torsion of the flagellar area (figure not shown). The sulcal area has the shape of a funnel (Figs. 2C, 4B, 5A and B) from which the longitudinal flagellum radially emerges from the cell. The anterior edge of Sp, together with a fold near the left end of 5‴, forms like a lip that enhances the funnel shape of the sulcal area (Fig. 5A and B).

The hypotheca is composed by five postcingular plates and two antapical plates in addition to Sp. The biggest plates of the postcingular series are 3‴ and 4‴ (Figs. 2C and 3B). Plate 1‴ is triangular (Fig. 2A and C). Plate 2‴ is trapezoidal being the dorsal part wider than the ventral part. Plate 3‴ is four sided and dorsally placed but slightly displaced to the left side (Fig. 2C). It overlaps all its neighbors. Plate 4‴ is five sided with a small and variable connection to the Sp plate (Figs. 2C and 3B). It occupies most of the right side of the postcingular area being the biggest of the postcingular series. Plate 5‴ is small and twisted having a fold near the ventral end (Fig. 5A and B). The most outstanding characteristic of the hypotheca is a narrow and small 2⁗ which is surrounded by big postcingular plates (Figs. 2C, 3B and 4B). This plate usually has parallel lateral sides but sometimes can it be wider to the ventral

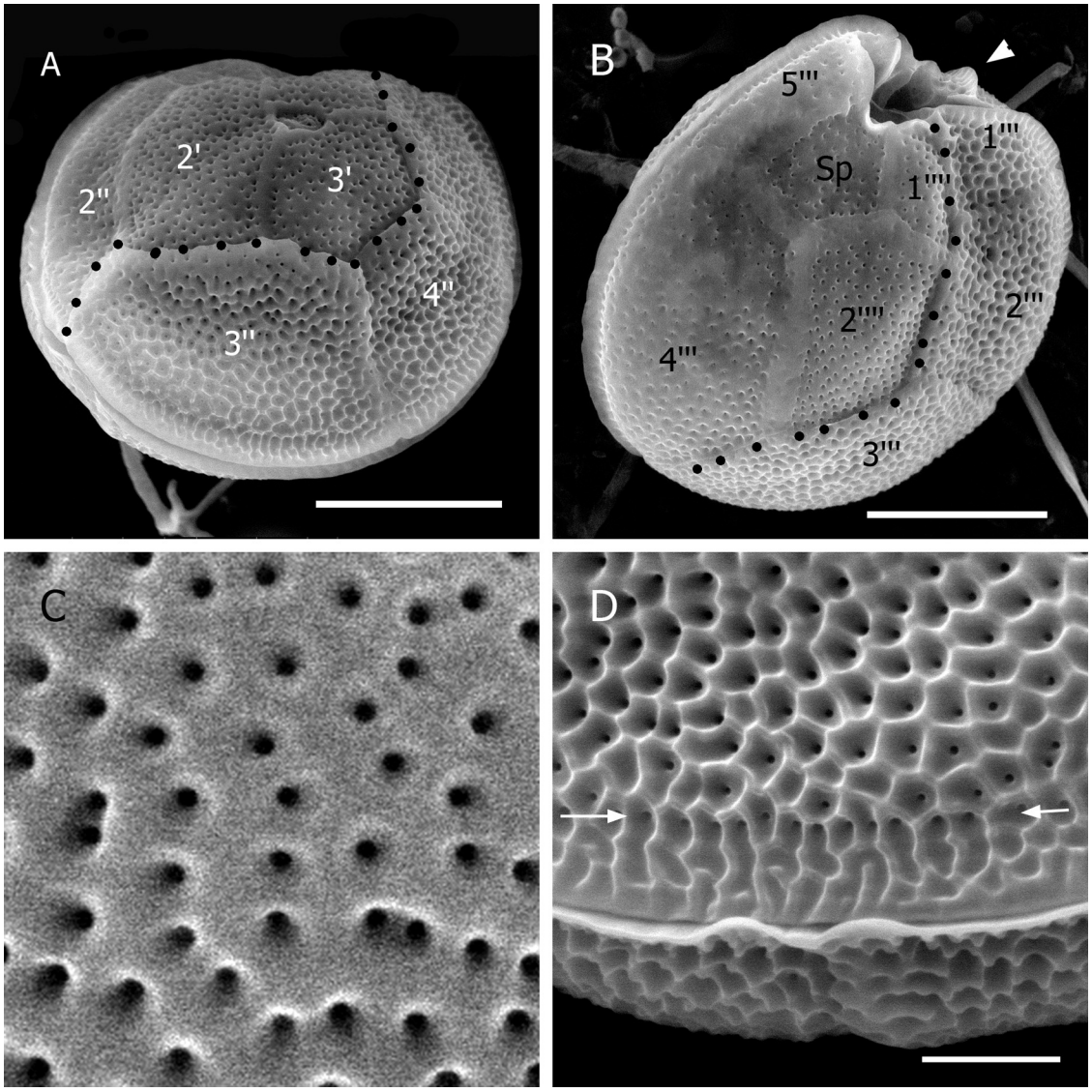

Fig. 4. SEM images of *Gambierdiscus balechii* (Strain VGO917). (A, B) Recently divided cells showing rugose and smooth thecal plates of epitheca and hypotheca. Dot lines indicate fission lines. Arrowhead shows one edge of Plate 1′. (C) A 10 × 10 μm square section of Plate 2′ showing pores pattern. (D) Detail of the ornamentation of a precingular plate. Arrows indicate line or marginal pores. Scale bars: (A, B) 20 μm, (D) 2 μm.

side. In the antapical series, 1′′′′ is more or less symmetrical to Sp and also contacts 1′′′, 2′′′, 2′′′′ (Figs. 2C, 3B and 4B) although the connection to 2′′′ is variable and can be reduced to a point where Plates 1′′′, 2′′′, 2′′′′ and 1′′′′ converge. Plate 2′′′′ contacts Sp, 1′′′′, 2′′′′, 3′′′, 4′′′ and sometimes 1′′′ in a point (Figs. 2C, 3B and 4B). It was never observed contact to 5′′′. Both precingular and postcingular series overlap the plates of the apical and antapical series respectively, and inside the series, dorsal plates overlap those more ventrally situated, starting from the dorsal side formed by Plates 3′′ and 3′′′ (Fig. 2). The cell division is oblique and one daughter cell keeps plates Po, the four apicals and 1′′ and 2′′ of the epitheca, and 1′′′, 2′′′ and 3′′′ of the hypotheca (Fig. 4A and B).

In addition to the above described morphology, which corresponds to the common cells observed in our cultures, other morphologies were observed. Cells in which Plates 2′′′, 3′′′, 4′′′ and 2′′′′, are in different plane and protruding their neighbor Plates 5′′′, Sp, 1′′′′ and 1′′′′ forming a step between these two groups of plates (Fig. 7A and B) were detected. It is remarkable that the division between these two parts of the hypotheca does not correspond with the division line of the cells which is oblique (Fig. 4A and B). Regarding small cells (about 35–40 μm), several different forms can be observed provided that the studied number of cells is large enough. Almost spherical cells without visible plates but fluorescent when stained with calcofluor. Globular cells with thin plates and relatively big Po, 1′ and 6′′ plates in comparison to the other plates (Fig. 7C and D). More flattened cells with a more conspicuous sulcus (Fig. 7F) and also with a relatively big Po plate. Moreover, among these different types some intermediate forms can be observed.

Non motile cells with appearance of cysts were observed both in cultures without N/P added and in stationary phase of the nutrient replete cultures. Of all isolated putative cysts, a range between 23 and 33% germinated. Morphology of these cysts was the same as vegetative cells but they were non motile, non-flagellated and some of them presented a thick and prominent theca (Fig. 8A and B). The cytoplasm of the cysts presented numerous green and brown grains, even some were observed to have red pigmentation (Fig. 8A). Furthermore, the cytoplasm of a few of the cysts which germinated was shrunken with rounded shape inside the theca (Fig. 8B). Ecdysal cells were often observed and took place by means of the protruding of the cytoplasm through an opening at the cingulum level (Fig. 8C).

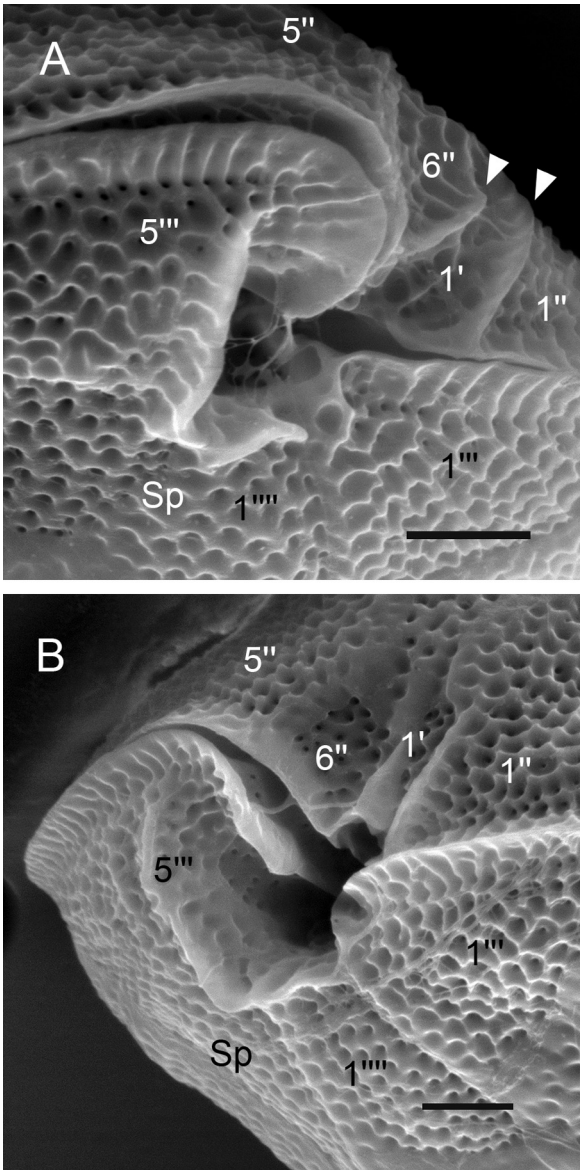

Fig. 5. SEM images of *Gambierdiscus balechii* (Strain VGO917). (A, B) Detailed sulcal area observed from right (A) and left sides (B). Arrowheads show keel-like edges of Plate 1′. Scale bars: 5 μm.

Gambierdiscus balechii has numerous and small chloroplasts. The pigment composition of *G. balechii* strain VGO920 was described by Zapata et al. (2012). It has a peridinin (Per)-containing chloroplast type widespread within photosynthetic dinoflagellates with chl c_2 and Per as major accessory pigments. HPLC pigment analyses revealed minor quantities of Chl c_1 in *G. balechii* (chl c_1/chl c_2 = 0.13) a pigment previously detected in the genus *Gambierdiscus* (Durand and Berkaloff, 1985). Diadinoxanthin (Diadino) and dinoxanthin (Dino) were also relevant pigments in the overall carotenoid pool. Pigment ratios to chl *a* for the main carotenoids and accessory chls were as follows: Per/chl *a* = 0.91, Diadino/chl *a* = 0.53 to Dino/chl *a* = 0.18 and chl c_2/chl *a* = 0.33.

3.3. Phylogenetic analyses

Phylogenetic trees based on LSUrRNA (D8–D10 regions), and SSUrRNA genes were elaborated (Figs. 9 and 10). Overall, both genes reconstructed similar relationships between clades of *Gambierdiscus*, including formally described species or ribotypes/sp. types based on molecular results from current available studies (Litaker et al., 2010; Nishimura et al., 2014; Xu et al., 2014). Sequences of *G. balechii* strains VGO917 and VGO920 clustered together as a separate group, sister to *G. pacificus* and *G. toxicus*. The D8–D10 phylogeny included a larger number of molecular clades/ribotypes and *G. balechii* branch was closer also, in addition to *G. pacificus*/*G. toxicus*, to *Gambierdiscus* sp. type 6 from Kiribati (Xu et al., 2014). In the LSU alignment (906 positions) average net genetic distances between *G. balechii* and its closest formally described species, *G. pacificus* (15 seqs.), *G. toxicus* (11 seqs.) and *G. scabrosus* (5 seqs.) were 0.015 and 0.021 and 0.027. Lowest values (0.005) were found relative to *Gambierdiscus* sp. type 6 (5 seqs.). If uncorrected genetic distances (p) are calculated, the values raise up to 0.011 between *G. balechii* and *Gambierdiscus* sp. type 6, and 0.021, 0.031 and 0.038 against *G. pacificus*, *G. toxicus* and *G. scabrosus*. Regarding partial SSU data (1591 positions), the average net genetic distances between *G. balechii* and its closest species, *G. pacificus* (10 seqs.), *G. scabrosus* (16 seqs.)and *G. toxicus* (14 seqs.)were 0.046, 0.063 and0.055, respectively. These represent higher values than the range observed among the latter three species (0.032–0.058).

3.4. Ecology and behavior

Gambierdiscus balechii was found in tidal ponds on rocky shores of volcanic origin in protected areas (Fig. 1). The cells were epiphytic over on *Ectocarpus* sp. but sampling cannot be considered as representative as it was done opportunistically. In comparison to other *Gambierdiscus* species in culture, *G. balechii* cultured in our laboratory was a very active species since swimming cells were commonly observed.

3.5. Toxicity

All the mice intraperitoneallly injected with liquid–liquid partition fractions (dichloromethane and aqueous methanol) obtained from the *Gambierdiscus balechii* strain VGO917 showed symptoms typical of CTXs, including loss of activity, piloerection, dyspnea, cyanosis, ataxia, tremor, convulsive jumping, hypothermia and death. Death happened by respiratory failure and differences were noted in the death times recorded comparing the two liquid-partition fractions. Thus, mice injected with aqueous methanol extracts, containing MTXs, died 52 min and 68 min after injection while mice injected with dichloromethane extracts, containing CTXs, died 29 min and 40 min after injection. Control mice injected with the Tween solution and the extracts of dichloromethane and aqueous methanol solvents did not show signs of toxicity and remained alive 24 h after intraperitoneal injection.

4. Discussion

4.1. Morphology

Litaker et al. (2009) divided the *Gambierdiscus* species in two groups according to the width of Plate 2′′′′. One group comprises the species with a wide 2′′′′, and another one those with a narrow 2′′′′. *G. balechii* belongs to the second group together with *G. pacificus, G. australes* and *G. excentricus*, *G. belizeanus* and *G. scabrosus*,. The first three species have a smooth theca very different from the heavily ornamented theca of and *G. balechii*, *G. belizeanus* and *G. scabrosus*. According to Nishimura et al. (2014) *G. belizeanus* can be distinguished from *G. scabrosus* by the shape of Plate 3′′ which is symmetric in the former (Litaker et al., 2009), and asymmetric in the later. Plate 3′′ of *G. balechi* is also asymmetrical (Fig. 4A), like in *G. scabrosus* from which it can be differentiate by the shape of Plate 2′, another critical morphological characteristic

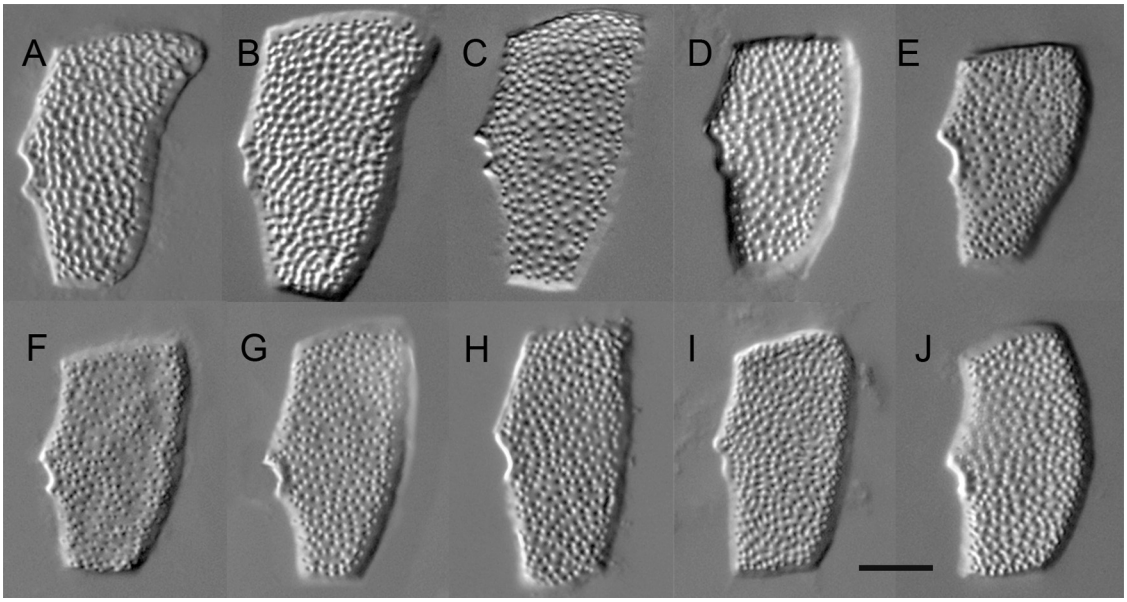

Fig. 6. LM images of *Gambierdiscus balechii* (Strain VGO917). (A–J) Variability of Plate 2′, from clear hatchet-shaped to almost rectangular. Scale bar: 10 μm.

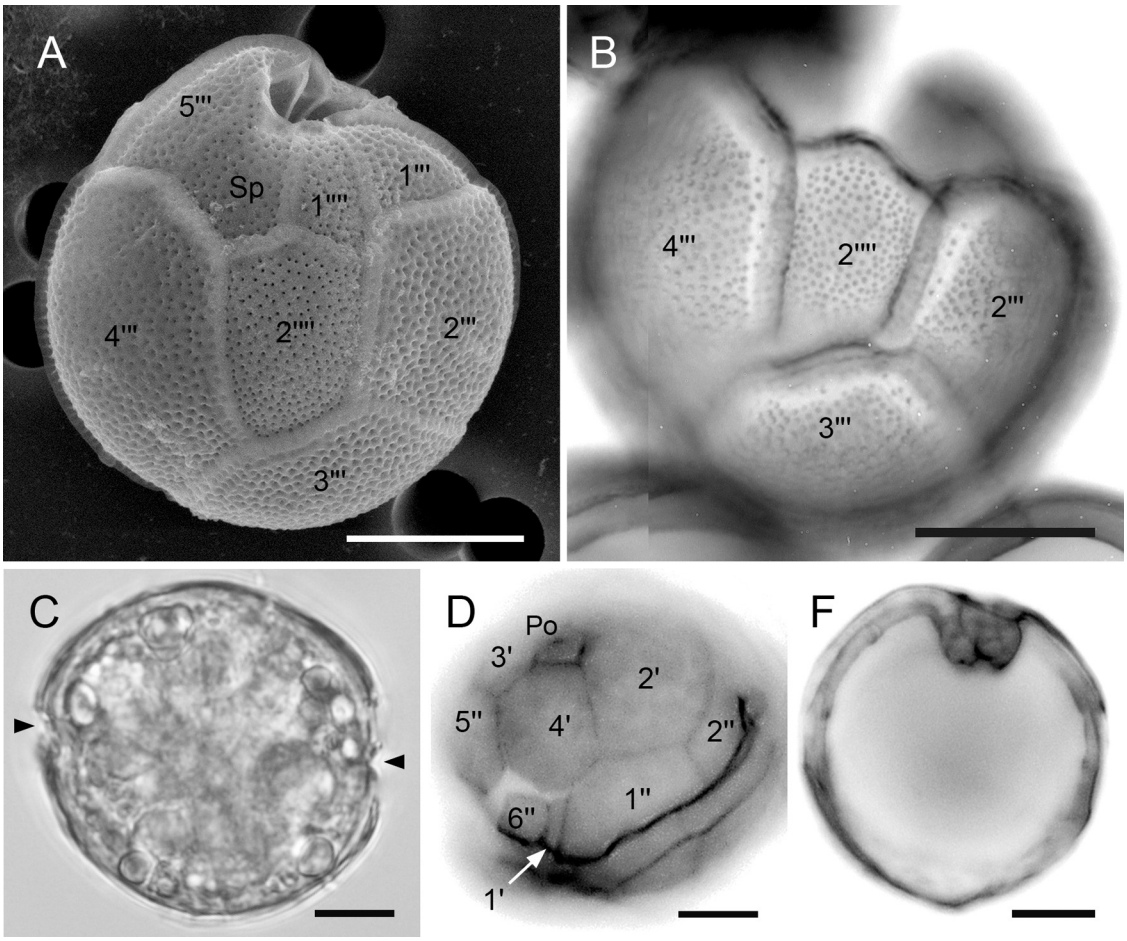

Fig. 7. Uncommon forms of *Gambierdiscus balechii* (Strain VGO917). (A) SEM image of the hypotheca with protruding plates. (B) Calcofluor-stained cell of the four hypothecal plates that are protruded. (C) Small globular shaped cell as indicated by the cingulum position. Arrowheads indicate the cingulum. (D). Calcofluor-stained small globular cell with thin plates showing Po, 1′ and 6′ big in relation to other plates. (F) Calcofluor-stained small globular cell showing a marked sulcal area. Scale bars (A, B): 20 μm, (C, D): 10 μm.

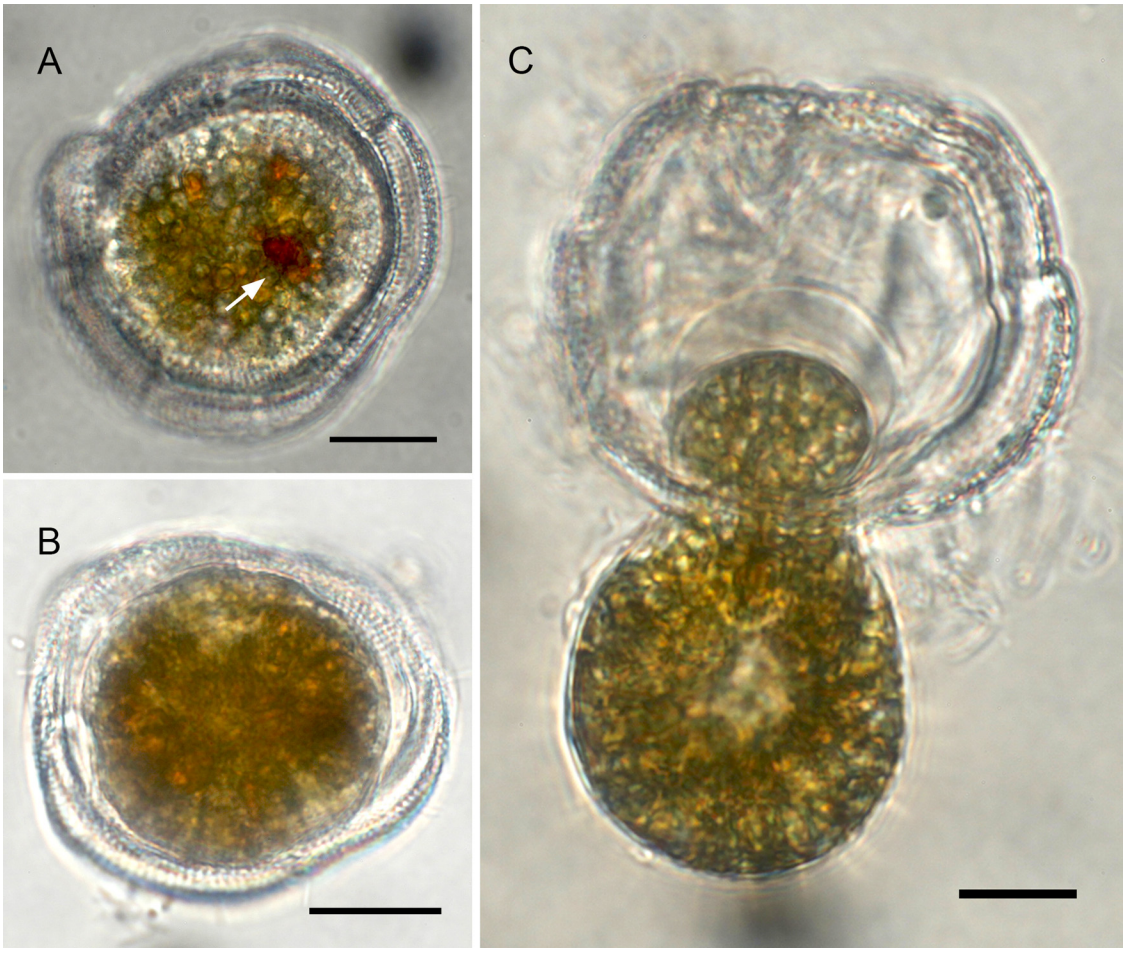

Fig. 8. *Gambierdiscus balechii* cysts (A) cyst showing a red pigmentation (arrow). (B) cyst with shrunken rounded cytoplasm inside the theca. (C) Cell during ecdysis. Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

identified by Litaker et al. (2009) for *Gambierdiscus* species classification together with the shape of Plate 2′′′′, According to the *G. scabrosus* original description (Nishimura et al., 2014) its Plate 2′ is rectangular while in *G. balechii* is usually hatchet-shaped (Fig. 6). When many *Gambierdiscus* cells are examined, as it was done in this work, a high morphological variability on the shape of critical plates can be observed. The average ratio between the 2′/1″ to 2′/3″ suture lengths observed in *G. balechii* was 0.64 (s = 0.14, n = 72) but the values range was from only 0.30, which is clearly hatchet-shaped, to 0.89 which is higher than what is considered rectangular in other species. Hence, the shape of Plate 2′ has to be taken carefully and many cells have to be observed before arriving to a conclusion on the identification of a *Gambierdiscus* to species level. The asymmetry of Plate 3″ has the same problem as sometimes its sutures with the contiguous Plates 2″ and 4″ can be almost equal in length and hence symmetrical while in the same species can be clearly different.

The cells that have a step between ventral and dorsal plates in the hypotheca were observed in cultures but it cannot be considered as an artifact of cultures as this character was also observed in wild specimens of *Gambierdiscus excentricus* from Morocco (Ennaffah and Chaira, 2015).

The great size variability shown herein corresponds with that reported by Bravo et al. (2014) for the same strain. *Gambierdiscus balechii* is the first described species of the genus showing such a wide size range. This is due to the thorough study of cell sizes those authors performed based on 1315 specimens, which is much bigger than what is generally used in other species descriptions within this genus. Small cells have been probably overlooked in the *Gambierdiscus* literature because they appeared at very low concentrations in the cultures. Among all the small cells, the ones with a globular shape are those which present the most interesting morphological distinctive features. Further studies are needed in order to clarify and define their detailed morphological traits and function.

Regarding the cells initially described here as putative cysts, they should be considered as thecate cysts because of their resemblance to non motile vegetative cells and the fact that they germinated after their isolation in fresh medium. This type of cysts has been described in the literature for other genera and their surviving time goes from days to months (Bravo and Figueroa, 2014). Reports on *Gambierdiscus* cysts are very scarce in the literature. In cultures of *G. toxicus*, Hokama et al. (1996) observed cells similar to our cysts. They described cells with thick and translucent walls as well as dense, dark brown central cores which the authors interpreted as cysts. Although Durand-Clement (1987) also reported a temporary coccoid cyst from cultures of *G. toxicus*, no cysts of *Gambierdiscus* have been reported from nature so far. Indeed, the life-cycles of benthic ciguatera-related dinoflagellates, such as those included in the genera *Ostreopsis*, *Gambierdiscus* and *Prorocentrum*, are poorly understood. And the occurrence of a sexual cycle which likely underlies the size variability depicted herein for *G. balechii* has been reported very recently (Bravo et al., 2014). Therefore, it would not be surprising if some benthic dinoflagellates have a resting stage similar to the benthic vegetative one. And it still remained cryptic due to the difficulty to clarify it.

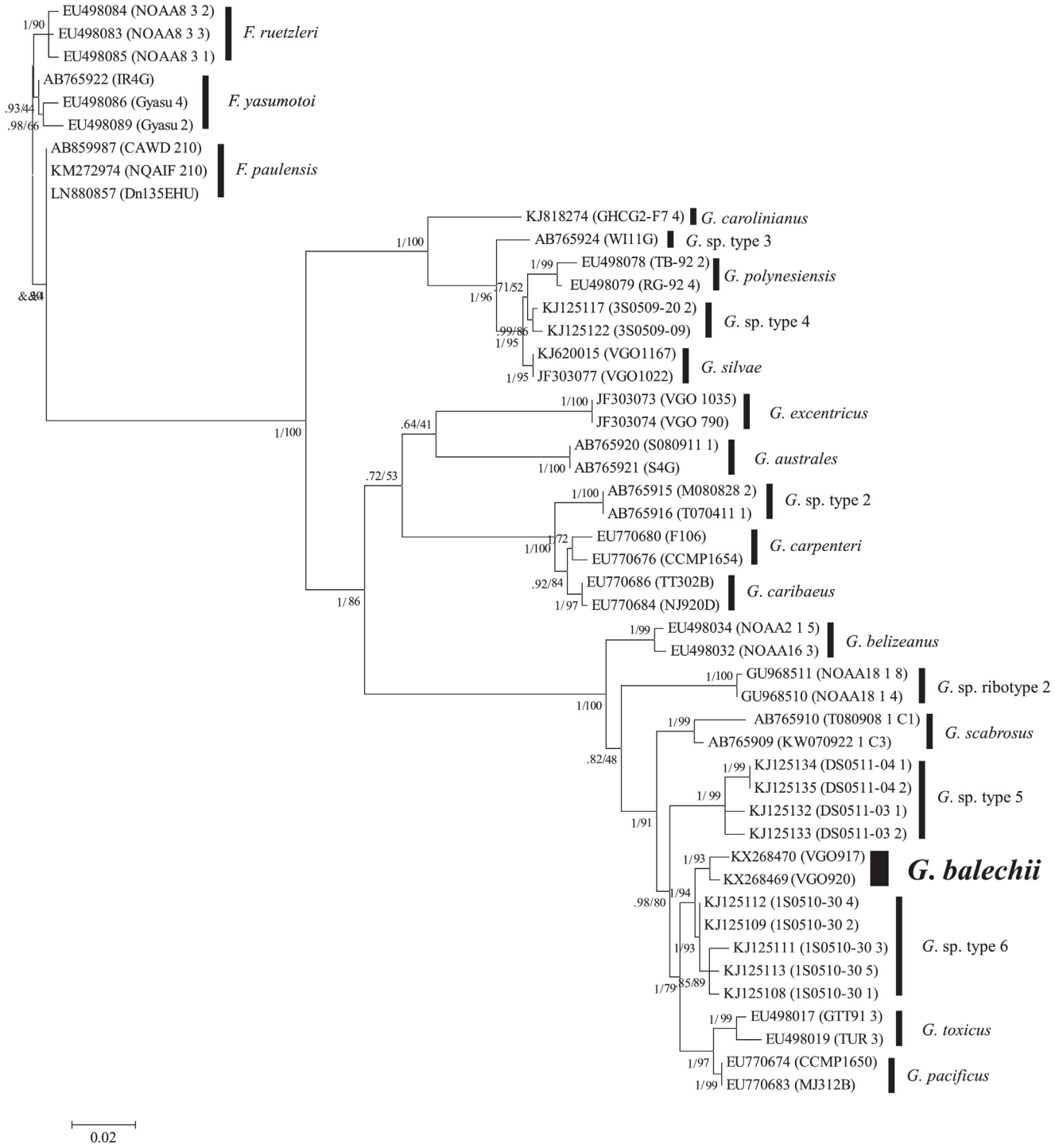

Fig. 9. LSUrRNA phylogeny (divergent domain D8–D10) showing the relationships between *Gambierdiscus balechii* and other *Gambierdiscus* species/phylotypes. GenBank Acc. Nos. and strain names are detailed in each case. Internal node supports indicate posterior probabilities (Bayesian analyses) and bootstrap values (Maximum Likelihood). Hyphens denote bootstrap values <60.

4.2. Phylogeny

The taxonomic complexity in the genus *Gambierdiscus* rapidly increased in the last years with the addition of new species. Nowadays, after the transfer of former *G. yasumotoi* and *G. ruetzleri* species to the new genus *Fukuyoa* (Gomez et al., 2015), *Gambierdiscus* is constituted by 12 formally described species, including the present report of *G. balechii* (see Section 1).

Among the *Gambierdiscus* clades first characterized on the basis of molecular data (mainly LSUrRNA), two of these, *Gambierdiscus* sp. ribotype 1 (Litaker et al., 2009) and *Gambierdiscus* sp. type 1 (Nishimura et al., 2013) were recently described as the new species *G. silvae* and *G. scabrosus*, respectively (Fraga and Rodríguez, 2014; Nishimura et al., 2014).

Yet, six *Gambierdiscus* ribotypes (sp. types 2–6, sensu Nishimura et al., 2013; Xu et al., 2014; and *Gambierdiscus* ribotype 2, Litaker et al., 2009) retrieved from ribosomall sequences are pending of their confirmation as valid taxonomic species, their examination being dependent on the availability of living specimens and cultures. The phylogenetic relationships depicted in LSUrRNA and

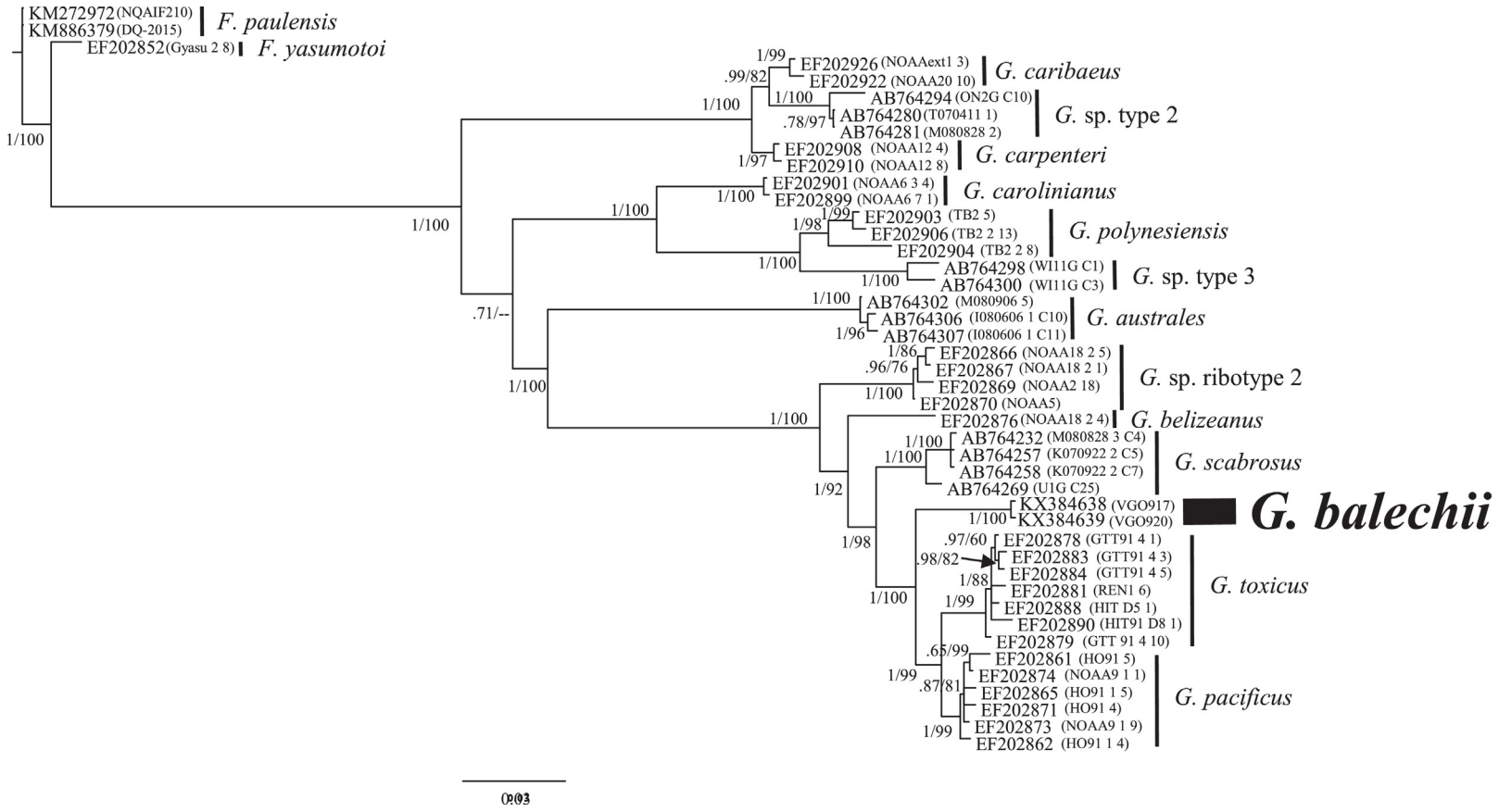

Fig. 10. SSUrRNA phylogeny showing the relationships between *Gambierdiscus balechii* and other *Gambierdiscus* species/phylotypes. GenBank Acc. Nos. and strain names are detailed in each case. Internal node supports indicate posterior probabilities (Bayesian analyses) and bootstrap values (Maximum Likelihood). Hyphens denote bootstrap values <60.

SSUrRNA phylogenies in our study placed *G. balechii* far enough from other formally described species such as *G. pacificus*, *G. toxicus* and *G. scabrosus*, sister also of two molecular ribotypes from Kiribati Islands (Pacific), *Gambierdiscus* sp. type 5 and specially *Gambierdiscus* sp. type 6 (Xu et al., 2014).

The genetic data presented here for two strains of *G. balechii* (VGO917 and VGO920) shows that their closest known relative is *Gambierdiscus* sp. type 6. The mean genetic distances calculated in our study (0.005) are in the lowest range for those reported between closely related *Gambierdiscus* species (such as *G. caribaeus*/*G. carpenteri* and *G. toxicus*/*G. pacificus*), or the now *Fukuyoa yasumotoi*/*F. ruetzleri* (Fraga and Rodríguez, 2014; Litaker et al., 2009). Nevertheless, it must be remembered from previous phylogenetic studies that the divergent domain D8–D10 yields smaller distances between pairs of *Gambierdiscus* species than those observed in D1–D3 sequences (Fraga and Rodríguez, 2014; Litaker et al., 2009).

In an intensive survey in coastal Japan (Nishimura et al., 2013), the sequencing of LSU rDNA (D8–D10) in 248 *Gambierdiscus* strains rendered a mean uncorrected genetic (p) distance within-species and between-species of 0.002 ± 0.002 and 0.121 ± 0.036, respectively. It must be noticed that the between-species limits were very variable depending on the particular pair of species; lowest range was ten-fold lower for *G. caribaeus*/*G. carpenteri* (0.009) and *G. pacificus*/*G. toxicus* (0.014) (Nishimura et al., 2013). Therefore, the uncorrected genetic distance determined in our study between *G. balechii* and other formally described species is well above the minimum range for other species in the genus. Nevertheless, its genetic similarity against *Gambierdiscus* sp. type 6 deserves further exploration using other genetic similarity against markers (namely D1–D3 LSUrRNA), that are expected to reveal larger differences than those already demonstrated in the D8–D10 phylogeny. In the case of SSU sequences, genetic distances calculated from the partial phylogeny in this study cannot be directly compared to those by Nishimura et al. (2013) based on full length SSU fragments. These authors mentioned a between species limit larger than that from LSU (0.139 ± 0.042). In the present work, the range of uncorrected genetic distances between *G. balechii* and the sister species *G. pacificus*, *G. scabrosus* and *G. toxicus* (0.026–0.032), would lie also over the median value between *G. pacificus*/*G. toxicus* (0.022; (Nishimura et al., 2013). In conclusion, the above mentioned results demonstrate that LSU and SSU phylogenies support the existence of *G. balechii* as a separate species, distinct from other formally described taxa (in particular *G. pacificus* and *G. scabrosus*). Only the sister ribotype *Gambierdiscus* sp. type 6 should be further examined to clarify its taxonomic position against the newly described species.

4.3. Toxicity

Results from MBA revealed toxic activity of *G. balechii* typical of CTXs. This finding has considerable interest because among other closely related species in the genus, only *G. scabrosus* has been shown to display CTX and MTX activity in MBA analyses (Nishimura et al., 2014), whereas *G. pacificus* and *G. toxicus* appear to be virtually non-toxic (Parsons et al., 2012). Therefore, the positive MBA results for *G. balechii* suggest that this species could be involved in CFP in Indonesian waters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2016.06.004.

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