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Title: Gambierdiscus excentricus sp. nov (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean)

Article Type: Original Research Article

Keywords: Gambierdiscus excentricus; benthic dinoflagellates; ciguatera; Canary Islands; ciguatoxin; maitotoxin

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Abstract: A new benthic toxic dinoflagellate is described from the Canary Islands, Spain. Gambierdiscus excentricus sp. nov. was isolated from seaweeds growing in tidal ponds and was observed in winter and summer. Its' morphology was studied by means of Light Microscopy (LM) and Scanning Electron Microscopy (SEM); G. excentricus is a lenticular species having a Po plate ventrally displaced in relation to other species of the genus Gambierdiscus. Phylogenetic trees from large subunit (LSU) of ribosomal RNA gene LSUrDNA sequences displayed a topology confirming that G. excentricus clustered in its' own group, separated from the rest of Gambierdiscus species and with G. australes as its closest relative. Pigment composition studied from G. excentricus cultures, included peridinin, as the major carotenoid, chlorophyll a and the accessories accessory chlorophylls c1 and c2. The Neuroblastoma cell-based assays for ciguatoxins (CTX) and maitotoxin (MTX) allowed identifyingconfirmed G. excentricus as a CTX- and MTX-like compounds producer. The finding of a toxic species of Gambierdiscus in the Canary Islands may explain the recent reported cases of ciguatera in the area.

Cover Letter

MINISTERIO
DE CIENCIA
E INNOVACIÓN

INSTITUTO ESPAÑOL
DE OCEANOGRAFIA

CENTRO
OCEANOGRÁFICO
DE VIGO

Vigo, 29 June 2011

Dr. Sandra E. Shumway
Department of Marine Sciences
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Dear Sandy,

I have made all the changes recomended by the both reviewers plus some additional minor corrections that I found neccessary.

Best regards,

Santi

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Dear Editor,
These are the answers to the referees comments

Review of "*Gambierdiscus excentricus* sp. nov (Dinophyceae), benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean)"

Referee 1

General comments:

Pg 7, lines 153-154. This Chelex extraction procedure was first used and described for Gambierdiscus by Richlen and Barber (2005), which should be cited here, not Litaker et al., 2010.

Richlen, M.L. and Barber, P.H. (2005) A technique for the rapid extraction ofmicroalgal DNA from single live and preserved cells. Molecular ecology notes 5: 688-691.

CHANGE DONE

'Section 2.1 - Were cultures eventually transferred to tubes or flasks?

INFORMATION ADDED

Section 2.6 - Were the PCR products cloned? If so, include here.

NO, THE FINAL SEQUENCES USED IN THIS WORK WERE NOT CLONED, WE INCLUDED ONLY THESE FROM SINGLE CELL PCR. WE ELIMINATED THE REFERENCE TO THE CLONING PROCEDURE IN THE METHODS SECTION.

Section 2.9 - Why were culture conditions here different than in Section 2.1?

MORPHOLOGICAL AND TOXINOLOGICAL STUDIES WERE DONE IN DIFFERENT LABORATORIES WITH DIFFERRENT ROUTINE CULTURE CONDITIONS WHICH DID NOT AFFECT THESE RESULTS.

Figure 6 - This figure is shown, but then barely mentioned in the manuscript. Please include further mention or discussion of this figure.

A REFERENCE TO THIS FIGURE WAS ADDED IN LINE 514 OF THE ORIGINAL MANUSCRIPT

Editorial comments:

Abstract - spell out abbreviations in abstract (e.g., LM, SEM)

CHANGE DONE

Line 29 - accessory not accessories

CHANGE DONE

Line 23 - and all subsequent instanees - its not its'

CHANGE DONE

Line 31 - suggest using "confirmed " instead of"allowed identifying"

CHANGE DONE

Line 67 - "making it necessary. ..."
CHANGE DONE

Line 116 - "following a modified method (Figueroa et al., 2010)
CHANGE DONE

Line 128 - "Scanning Eleetron Microscopy"
CHANGE DONE

Lines 130-135 - Spell out abbreviations.
CHANGE DONE

Line 167 - define LSU, and LSU rDNA subsequently
CHANGE DONE

Line 280 - this sentence seems to be missing "during 24 h ofprevious exposure"
CHANGE DONE. A NEW PARAGRAPH WAS WRITTEN.

Line 281 - "as described previously (Caillaud et al. . . . . .)
CHANGE DONE

Line 283 - "For the detection of MTX -like compounds....."
CHANGE DONE

Lines 287-288 - I would revise to read "After exposure of Neuro-2a cells to standard or *G. excentricus* solutions for the determination of CTX-like and MTX-like toxicity respectively, toxic effects...."
CHANGE DONE

Line 291 - "as described in Manger et al. (1993).
CHANGE DONE

Line 336 - Simply title section "Etymology"
CHANGE DONE

Line 396 - "chloroplasts radially dispersed...."
CHANGE DONE

Line.411 -...:. "sampling cannot be considered as representative, as it was done opportunistically. "
CHANGE DONE

Line 413 - "non motile, and usually the only appreciable movements are thebeating of..."
CHANGE DONE

Line 416 - "daughter cells usually appeared close to one another after division." Line 433 - "to inspect differences. . . "

CHANGE DONE IN LINE 424

Line 433 - "larger than that....."
CHANGE DONE

Line 429 - delete "instead"
CHANGE DONE

Line 432 - "sister group in the analyses. The distance between G. excentricus and G. australes is also larger than that calculated between G. toxicus vs G. belizeanus"
CHANGE DONE

Line 435; lines 555-561 "The D8-DIO sequence ofstrain VGO1022 was placed in a separate clade which included two sequences from Gambierdiscus "ribotype 1", as defined by Litaker et al. (2010)." 1 think these data also suggest that Gambierdiscus "ribotype 1" is probably *G. polynesiensis*.
CHANGE DONE

Line 473 - insert comma before which
CHANGE DONE

Lines 477-481 - can this be broken up into two sentences?
CHANGE DONE

Lines 483-454 - please c1arify.
I PRESUME ARE PAGES 483-484. CHANGES HAVE BEEN DONE IN THE WHOLE PARARGRAPH TO CLARIFY IT

Line 514 - "Moroccan"
CHANGE DONE

Line 517 - fix citation
CHANGE DONE

Line 518 - "in which this ratío is about 1.5, as the other discoid Gambierdiscus species”.
CHANGE DONE

Line 524 - insert comma before which
CHANGE DONE

Lines 525-526 - please clarify?
CHANGES DONE TO CLARIFY IT

Line 529 - this was also observed by Faust (1995) and Richlen et al. (2008)
CHANGE DONE

Line 530-533 - I would use "discounted" instead of"discarded"
CHANGE DONE

Lines 525-527 - Please clarify.

CHANGE DONE

Line 543 - "discount further. ..."
CHANGE DONE

Line 566 - Please rephrase this sentence
CHANGE DONE

Line 570 - "composition; however,..."
CHANGE DONE

Line 599 - "may not" repeated twice
CHANGE DONE

Lines 631-635 - sentence is somewhat confusing - please rewrite or split into two . sentences.
CHANGE DONE

Line 723 - reference typo
REFERENCE CORRECTED

Line 727 - reference typo
REFERENCE CORRECTED

Line 731-32 - reference typos .
REFERENCE CORRECTED

Une 852 - reference typo .
REFERENCE CORRECTED

Line 874 - (b) Antapical view?
CHANGE DONE

Une 897 - "among descendants"
CHANGE DONE

Figure 5 - Please make sure that fue figure labels use the same size and include the orientation of these cells in fue figure Ilegend.
FIGURE AND LEGEND CORRECTED

Referee 2

The taxonomic portion of this paper needs to be strengthened by increasing the number of figures. How much variability was observed in the sizing of these key plates used to determine this new species? From Figure 5, it appears to be much variability in general cell shape. Are the characters used to describe this species real?
GENERAL SHAPE IS VARIABLE ACCORDING TO THE AGE OF THE CELL, BUT WHAT IT IS IMPORTANT TO DIFFERENCIATE THIS SPECIES

FROM OTHER IS THE SHAPE AND PROPORTIONS OF PARTICULAR PLATES. IN THIS CASE, THE UNIQUE SECOND APICAL AND A NARROW SECOND ANTAPICAL. FIGURE 6 SHOWS EIGHT EXAMPLES OF SHAPE VARIABILITY OF THE CHARACTERISTIC SECOND APICAL PLATE.

The main weakness of the manuscript is the measurement of toxicity. Gambierdiscus cells were extracted with MeOH without partitioning to recover MTX and CTX separately. The authors need to provide further details on the procedure used and results obtained to support their toxin data.

WE AGREE WITH THE AUTHOR THAT SEPARATING BOTH TOXINS THROUGH EXTRACTION AND PARTITION PROCEDURES CAN CONTRIBUTE TO QUANTIFY MTX AND CTX. THIS APPROACH WAS CONSIDERED BUT, DUE TO THE LIMITED AMOUNT OF BIOMASS, AND AFTER SOME ATTEMPTS, THE APPORACH DESCRIBED IN THE MANUSCRIPT WAS CHOSEN. IT WILL BE NECESSARY TO PRODUCE MORE *G. EXCENTRICUS* BIOMASS TO OPTIMIZE SUCH SEPARATION,

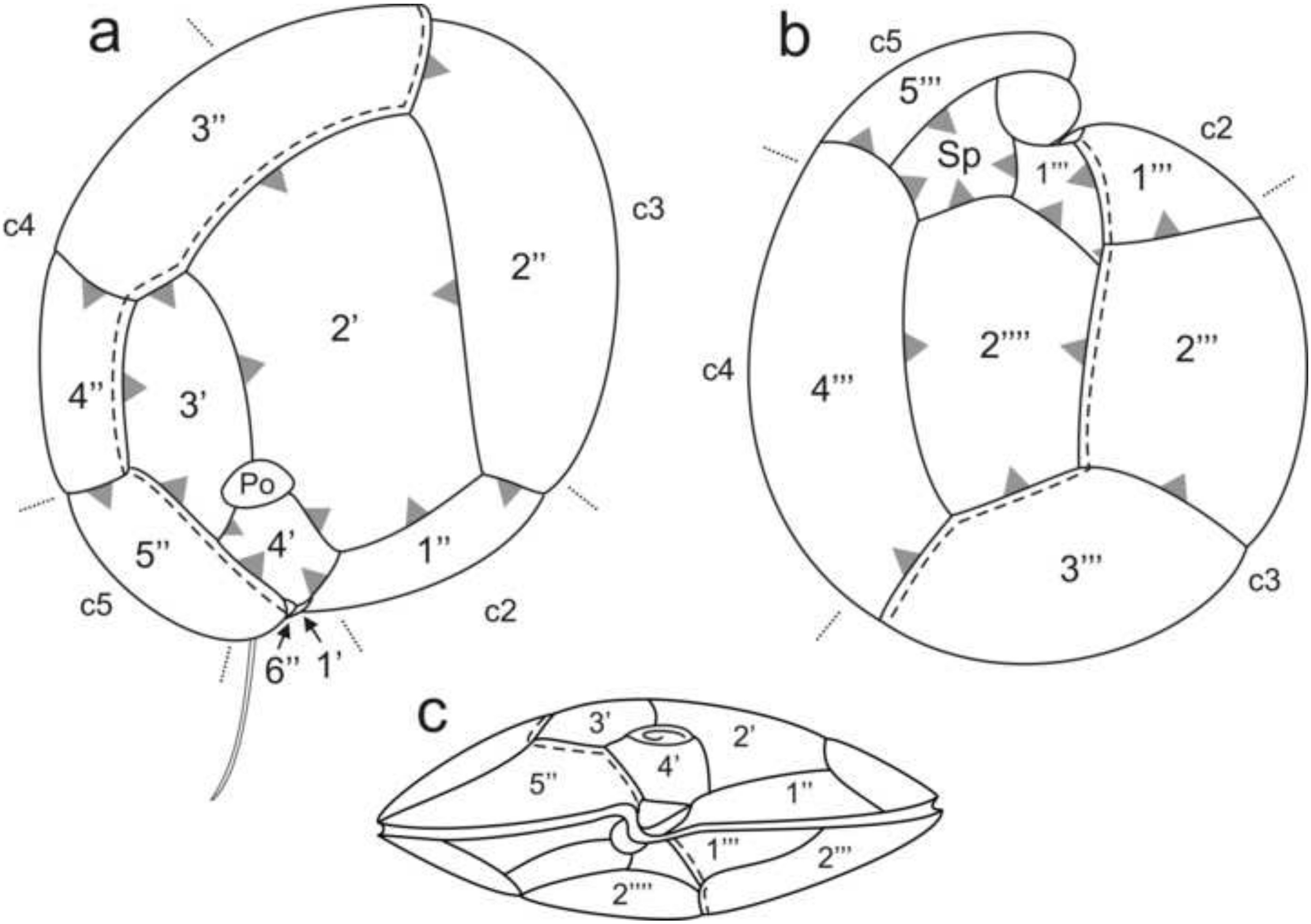
It is reported in the text that the extract was cytotoxic in the presence of ouabain and veratridine but there is no mention of the fact that the extract was not toxic in the absence of ouabain and veratridine at the dose tested, which is the only mean to show a CTX like specific activity. This will have to be mentioned in the manuscript. This is even more important that there was no partitioning and that MTX is cytotoxic with or without ouabain and veratridine.

WE CLEARLY IDENTIFY IN THE NEW VERSION OF THE MANUSCRIPT THE TOXIC RESPONSES IN IN THE ABSENCE AND IN THE PRESENCE OF OUABAIN AND VERATRIDINE. TABLE 3 HAS BEEN MODIFIED AND INCLUDES ADDITIONAL INFORMATION.

Regarding the MTX measurement: A brief description of the method would be valuable as this method is fairly new. For example, what was the dose ratio measured? (Was it close to 1 or similar to that measured for MTX standard?) - What was the quantity of extract added in cell equivalent/mL? (Was it similar to that added for CTX measurement?).

WE HAVE INTRODUCED CHANGES IN THE MANUSCRIPT

Graphical Abstract



***Highlights**

Two species of dinoflagellate *Gambierdiscus* have been found in the Canary Islands.
We describe *Gambierdiscus excentricus*, a new species of benthic dinoflagellate.
G. excentricus is toxic and produces ciguatoxins and maitotoxins.
G. excentricus could be the cause of ciguatera in the Canary Islands.

Gambierdiscus excentricus sp. nov (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean)

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Abstract

A new benthic toxic dinoflagellate is described from the Canary Islands, Spain. *Gambierdiscus excentricus* sp. nov. was isolated from seaweeds growing in tidal ponds and was observed in winter and summer. Its morphology was studied by means of Light Microscopy (LM) and Scanning Electron Microscopy (SEM); *G. excentricus* is a lenticular species having a Po plate ventrally displaced in relation to other species of the genus *Gambierdiscus.* Phylogenetic trees from large subunit (LSU) of ribosomal RNA gene sequences displayed a topology confirming that *G. excentricus* clustered in its' own group, separated from the rest of *Gambierdiscus* species and with *G. australes* as its closest relative. Pigment composition studied from *G. excentricus* cultures, included peridinin, as the major carotenoid, chlorophyll *a* and the accessory chlorophylls c_1 and c_2. The Neuroblastoma cell-based assays for ciguatoxins (CTX) and maitotoxin (MTX) confirmed *G. excentricus* as a CTX- and MTX-like compounds producer. The finding of a toxic species of *Gambierdiscus* in the Canary Islands may explain the recent reported cases of ciguatera in the area.

Keywords

Gambierdiscus excentricus; benthic dinoflagellates; ciguatera; Canary Islands; ciguatoxin; maitotoxin.

1. Introduction

Ciguatera fish poisoning (CFP) is a food-borne disease widespread in tropical and sub-tropical marine areas affecting mainly the Caribbean Sea, Polynesia and other areas in the Pacific, Indian Ocean (Lewis, 2006) although it has been also recently reported in the Canary Islands (Spain), a temperate area (Pérez-Arellano et al., 2005) and in Madeira (Gouveia et al., 2010; Otero et al., 2010) . CFP occurs after consumption of fish contaminated with ciguatoxins (CTXs) (Alfonso et al., 2005) but presence of additional toxins has been also proposed and cannot be discarded (Anderson and Lobel, 1987). Marine benthic dinoflagellate of the genus *Gambierdiscus* Adachi et Fukuyo (Adachi and Fukuyo, 1979; Yasumoto et al., 1977) are responsible for the production of CTXs further transmitted through the food web among reef fishes (Alfonso et al., 2005). The same genus may also produce other toxins i.e maitotoxins (MTXs), gambierol and gambieric acid. MTXs 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 (Alfonso et al., 2005).

The genus *Gambierdiscus* had been considered monospecific for fifteen years with *Gambierdiscus toxicus* Adachi & Fukuyo (Adachi and Fukuyo, 1979) as the only described species, a thecate gonyaulacoid dinoflagellate anteroposteriorly compressed with lenticular shape. The original plate formula was defined as Po, 3’, 0a, 7’’, 6c, 8s, 6’’’, 1p, 1’’’’ (Adachi and Fukuyo, 1979). *G. belizeanus* Faust (Faust, 1995) was the second species of the genus and it is easily distinguished from *G. toxicus* in having an ornamented theca and some differences in relation to the shapes of plates. The third species being described was *G. yasumotoi* Holmes (Holmes, 1998), a species very different from the other in being globular instead of discoid. Later, the diversity of the genus was found to be much higher than expected and recently seven new species have been added to the genus (Chinain et al., 1999; Litaker et al., 2009) based on morphology and on genetics which helped to find semicryptic species (Litaker et al., 2009; Richlen et al., 2008). Genetic sequences enabled even to find that the original description of *G.*

toxicus was based on more than one species making it necessary to describe a new epitype of the species (Litaker et al., 2009).

The Canary Islands archipelago (Fig. 1) is bathed by the Canary Current which is the eastern boundary current of the subtropical North Atlantic gyre. The area is characterized by low biomass and very oligotrophic waters where nutrients are depleted in summer (Cianca et al., 2007; Neuer et al., 2007). In this paper we describe *Gambierdiscus excentricus*, a new toxic dinoflagellate found in the Canary Islands coasts and report the presence of *Gambierdiscus* cf. *polynesiensis* in the same area. In addition to the taxonomic description of *G. excentricus*, production of toxins was examined.

2. Materials and methods

2.1. *Source of specimens and culture conditions*

Samples were collected at several locations in the Canary Islands' archipelago in the NE Atlantic Ocean (Fig. 1): 1) Punta Hidalgo, a rocky shore on the north coast of Tenerife, (28º 34' N, 16º 19' W) on March 28th, 2004; 2) Charca del Conde, La Gomera (28º 05' N, 17º 20' W) on November 15, 2005; and 3) Playa Las Cabras, La Palma (28º 29' N, 17º 49' W) on March 13, 2010. Samples of small mixed seaweeds and turf in grooves were collected from tidal pools on the rocks during low tide or from drifting seaweeds very near the coast and placed in plastic bottles and shaken. Afterwards, the gross particles were removed and the remaining seawater was used for cell isolation. Isolation was carried out by a capillary pipette with the aid of a ZEISS Invertoscop D microscope (Carl Zeiss AG, Germany). Isolated cells were incubated in 96 microwells 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 psu and incubated at 25 ºC and a photon irradiance of about 90 µmol $m^{-2}\cdot s^{-1}$ of PAR measured with a QSL-100 irradiameter (Biospherical Instruments Inc. San Diego, CA, USA) and at a 14:10 L:D photoperiod. The cultures were transferred to 100 mL Erlenmeyer flasks and to 50 mL polystyrene tissue culture flasks. The cultured strains VGO790, VGO791 and VGO792 were from Tenerife Island and VGO1035 from La Palma Island and all were deposited at the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo.

2.2. *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. 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. When the depth of field was not enough for the whole object, several pictures were taken at a series of different foci and were then merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). Cell size was measured by bright field LM on living cells on calibrated digital photographs. Cells stained with Fluorescent Brightener 28 (Sigma-Aldrich, St Louis, MO, USA) were also observed with a Leica TCS SP5 confocal microscope with UV light (Leica Microsystems GmbH, Wetzlar, Germany) at the CACTI facilities (Universidade de Vigo, Spain). The nucleus was stained using SYBR Green (Molecular Probes, Eugene, OR, USA) following a modified method (Figueroa et al., 2010) as follows: A 10 mL aliquot of culture was fixed with 0.5% paraformaldehyde for 10 min and washed in PBS pH7.0 (Sigma-Aldrich, St.Louis, USA) by centrifugation at 1200g during 10 min. Chlorophyll was extracted by resuspending the pellet in 5 mL of cold methanol and then storing the suspension overnight in the refrigerator. The cells were then washed twice in PBS (pH 7.0) as described above and the pellet was stained with a 1:200 solution of SYBR green in PBS 0.01M (pH7.4) and observed in a Leica DM LA epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) with blue excitation and photographed with a Canon EOS D60 (Canon Inc., Tokyo, Japan) digital camera. The autofluorescence of the chloroplasts was photographed with a Canon EOS 5D Mark II (Canon Inc., Tokyo, Japan) digital camera.

2.3. *Scanning Electron Microscopy*

Five mL of exponentially growing cultures were fixed with glutaraldehide (GTA) at a final concentration of 4%. After two hours 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% hexamethyldisilazane (HMDS). 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 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

2.4. *Nomenclature*.

In this study, a modified Kofoid tabulation system (Kofoid, 1909) as described in (Besada et al., 1982) was followed to name the plates therefore allowing comparisons with other genera. The main differences are: In the epitheca, we considered as the first apical plate (1’) what most of the authors consider as first precingular plate (1’’) and in the hypotheca, second antapical plate (2’’’’) instead of 1p, and sulcal posterior (S.p.) instead of second antapical (2’’’’) (More details in section 4.1.). The terms “length” as apical/antapical distance, “width” as transdiameter and “depth” as dorso/ventral distance were used for the cells dimensions.

2.5. DNA extraction.

Single cells of *Gambierdiscus* were picked up with a micropipette, washed in three distilled water droplets, and stored overnight at -80 ºC in 200 µL tubes. Prior to direct PCR on these single cells, samples were heated at 94 ºC during 1 min in the thermal cycler. DNA extracts were also used for amplification following a Chelex extraction procedure (Richlen and Barber, 2005) from 2-5 cells of *Gambierdiscus*. Single cells were isolated from cultures and washed in sterile dH_20 before being placed in a 200 µL tube containing 10 µL of 10x PCR buffer. The tubes were stored overnight at -80 ºC. Prior to DNA extraction, the tubes were centrifuged to settle the cells and 30 µL of 10% Chelex 100 (Bio-Rad, Hercules,California, USA) in dH_2O was added. The tubes were boiled at 95 ºC in a Eppendorf Mastercycler EP5345 thermocycler (Eppendorf AG, New York, USA) for 10 min, then vortexed. The boiling and vortex steps were done twice and samples were centrifuged (13,000 rpm for 1 min). The supernatants were transferred to clean 200 µL tubes avoiding to carryover the Chelex beads. Samples were stored at -20 ºC until PCR amplification.

2.6. PCR amplification and DNA sequencing

The D1-D3 and D8-D10 regions of the LSU gene were amplified using the pairs of primers D1R/LSUB (5’-ACCCGCTGAATTTAAGCATA-3’/5’-ACGAACGATTTGCACGTCAG-3’ (Litaker et al., 2003; Scholin et al., 1994), and FD8/RB (5’- GGATTGGCTCTGAGGGTTGGG-3’/5’-GATAGGAAGAGCCGACATCGA-3’ (Chinain et al., 1999), respectively, to produce readable sequences ranging 820-900 nucleotides. The amplification reaction mixtures (25 µL) contained 4 mM $MgCl_2$, 0.5 pmol of each primer, 0.8 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, California, USA), and 2 µL from the single cell Chelex extractions. The DNA was amplified in a Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA) following the conditions detailed elsewhere (Chinain et al., 1999; Litaker et al., 2003). A 10 µL aliquot of each PCR reaction was checked by agarose gel electrophoresis (1% TAE, 50 V) and SYBR Safe DNA gel staining (Invitrogen, California, USA).

The PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA). Purified DNA was sequenced using the Big Dye Terminator v3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) and migrated in an AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidade de Vigo, Spain).

The D1–D3 and D8–D10 sequences obtained in this study were deposited in GenBank (GenBank ID: HQ877874), (GenBank ID: JF303063-GenBank ID: JF303077).

2.7. Phylogenetic analyses.

LSU sequences were inspected and aligned using CLUSTALW multiple alignment in Bioedit (Hall, 1999). Uncorrected genetic distances (p; number of substitutions per site) were calculated for the original alignments using DNAdist v3.5c in Bioedit. Poorly aligned positions and divergent regions were checked using the GBLOCKS software (Castresana, 2000). A final number of 363 and 525 bases (38% and 66% of the original positions in D1-D3 and D8-D10, respectively) were saved by GBLOCKS. The final alignments were converted to nexus files using SeqVerter 2.0 (GeneStudio, Inc., USA).

The phylogenetic relationships were determined using a General Time Reversible model (GTR) in MrBayes v3.1 (Huelsenbeck and Ronquist, 2001). The program parameters were statefreqpr = dirichlet (1,1,1,1), nst = 6, rates = invgamma, nswaps = 1. The phylogenetic analyses involved two parallel analyses, each with four chains. In both parallel analyses there was one cold and three incrementally heated chains, where the heat of the ith chain is B =1/[1 + (i 2 1)T] and T=0.02. Starting trees for each chain were selected randomly using the default values for the MrBayes program. The corresponding number of unique site patterns was 189 and 158 in D1-D3 and D8-D10 analyses.

The number of generations used in these analyses was 400,000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization (‘‘burn-in’’ phase). All final split frequencies were less than 0.012. For comparative purposes, phylogenetic analyses were also conducted for each dataset after estimating different models of DNA substitution and associated parameters with Modeltest 3.7 (Posada and Crandall, 1998). Phylogenetic trees were obtained using a Tamura and Nei (1993) model with γ distribution (TrN + G) in PAUP 4.0b10 (Swofford, 2002) according with Modeltest 3.7 settings. Bootstrap values were estimated from 1000 replicates.

2.8. *Pigment analyses*.

Cultures were examined by light microscopy before carrying out HPLC pigment analysis to ensure the cells were healthy and presented good morphology (absence of alterations of the general structure). Cells were harvested 3 hours into the light cycle from cultures in exponential growth phase. Ten mL of culture were filtered onto Whatman GF/F filters (Whatman International Ltd. UK) under light vacuum. Filters were frozen immediately at -25 ºC, and analyzed within 12 hours. Frozen filters were extracted under low light in Teflon-lined screw capped tubes with 5 mL 90% acetone using a stainless steel spatula for filter grinding. The tubes were chilled in a beaker of ice and sonicated for 5 minutes in an ultrasonic bath. Extracts were then filtered through 25 mm diameter syringe filters (MFS HP020, 25 mm, 0.20 μm pore size, hydrophilic

PTFE,) to remove cell and filter debris. An aliquot (0.5 mL) of methanol extract was mixed with 0.2 mL of water and 200 µL was injected immediately. This procedure avoids peak distortion of early eluting peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to injection in an HPLC system (Latasa et al., 2001). Pigments were separated using a Waters (Waters Corporation, Milford, MA) Alliance HPLC System consisting of a 2695 separations module, a Waters 996 diode-array detector and a Waters 474 scanning fluorescence detector. Pigment separation was performed following previous work (Zapata et al., 2000), with a reformulated mobile phase A described below. The column was a C8 monomeric Waters Symmetry (150 x 4.6 mm, 3.5 µm particle-size, 100 Å pore-size). Eluent A was methanol: acetonitrile: 0.025 M aqueous pyridine (50:25:25 v/v/v). Eluent B was methanol: acetonitrile: acetone (20:60:20 v/v/v). Elution gradient was: (time: %B) t0: 0%, t22: 40%, t28: 95%, t37: 95%, t40: 0%. Flow rate 1.0 mL·min^{-1} and column temperature was 25 ºC. Solvents were HPLC grade (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). After checking for peak purity, spectral information was compared with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see (Jeffrey and Wright, 1997).

2.9. Toxin analysis.

Cultures of *G. excentricus* (strains VGO790, VGO791 and VGO792) were transferred to IRTA Laboratory where they were cultured in 1L Fernbach in a 33 practical salinity unit (psu) modified ES medium (Provasoli, 1968) at 24 ºC under a 12:12 light:dark regime with a photons flux rate of 80 µmol photon m^{-2} s^{-1} (QSL-2100 Radiometer, Biospherical instruments, San Diego, USA) and under permanent aeration. When cultures reached its stationary growth phase with cell densities of 1050, 2231 and 1217 cells mL^{-1} for strains VGO790, VGO791 and VGO792 respectively, cells were harvested through filtration using Whatman GF/F filters (Whatman International Ltd. UK). Filters were stored in absolute methanol at -20 ºC until toxin extraction.

For toxin extraction, GF/F filters were sonicated during 30 minutes at 38% amplitude (Sonics Vibracell, Newton, USA) in an extraction volume (Ve) of absolute methanol proportional to total cell density with Ve in mL equivalent to 10 x 10^6 cells. Methanol was further recovered after 5 minutes centrifugation at 4 ºC at 600g (Joan MR23i, Sant Herblain, France). This procedure was repeated one time with absolute methanol and twice with methanol:water (50:50, v:v) with the same Ve. Supernatants were pooled and evaporated until dryness at 40 ºC (Büchi R-200 or Büchi Syncore, Flawil, Switzerland). Extracts were finally dissolved in absolute methanol and kept at -20 ºC until analysis.

The Neuro-2a CBAs specific for CTXs (Manger et al., 1995) and MTXs (Caillaud et al., 2010c) were used for the determination of CTX- and MTX-like toxicity in *G. excentricus* crude extracts. Neuro-2a cells (ATCC, CCL131) were maintained in 10% foetal bovine serum (FBS) RPMI medium (Sigma, St Louis, MO, USA) at 37 ºC in a 5% CO_2 humid atmosphere (Binder, Tuttlingen, Germany) as previously described in (Cañete and Diogène, 2008). For experiments, cells were inoculated in a 96-well microplate at a density of 35,000 cells per well and incubated 24h before cytotoxicity assays under the same conditions as described for cell maintenance.

In order to specifically detect the presence of CTX-like compounds, Neuro-2a cells were first treated with 0.1 nM ouabain and 0.01 mM veratridine (V) (Sigma-Aldrich, St Louis, MO, USA) previous exposure of Neuro-2a cells to *G. excentricus* crude extracts during 24 hours as described previously (Caillaud et al., 2011; Cañete and Diogène, 2008). Sensitivity of the Neuro-2a cells to the presence of CTX was calibrated using a standard solution of Pacific type 1 CTX (CTX1B) provided by Dr. R.J. Lewis (The Queensland University, Australia).

For the detection of MTX-like compounds, Neuro-2a cells were first treated with 30µM SK&F 96365 (Sigma-Aldrich, St Louis, MO, USA) during 30 minutes previous exposure to *G. excentricus* crude extracts during 2.5 hours as described before (Caillaud et al., 2011; Caillaud et al., 2010c). The Neuro-2a CBA for MTX was calibrated using a MTX standard solution which was a generous gift from Prof. T. Yasumoto (Japan Food Research Laboratory, Japan).

After exposure of Neuro-2a cells to standards or *G. excentricus* crude extracts for the determination of CTX-like and MTX-like toxicity respectively, toxic effects were measured using the colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] MTT (Sigma-Aldrich, St Louis, MO, USA) cell viability evaluation (Mosmann, 1983) as described in Manger et al. (1993). Absorbance was read at 570 nm using an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA) and absorbance values were expressed in percentage of viability respect to its respective control (with and without O/V or SK&F 96365 treatment).

Results of cell viability were analyzed using the software Prism 4 (GraphPad, San Diego, CA, USA). A dose-response curve fit with sigmoid regression curve (with variable slope) was determined for each experiment and allowed estimating the concentration of *G. excentricus* extract or standards that inhibited 50% cell viability (IC_{50}) for each experimental condition (with and without O/V or SK&F 96365 treatment). IC_{50}s were further used as a toxicological parameter for the qualitative and quantitative estimation of the content of CTX- and MTX- like compounds produced by *G. excentricus*. Significant differences between means of IC_{50}s or toxin contents were analyzed using unpaired t-test (comparison of two means) and ANOVA (comparison of three or more means) with a 95% confidence level.

Production of CTX-like compounds by *G. excentricus* was identified when differences between IC_{50}s for O/V treated and non treated cells were significant ($p < 0.05$). When unspecific toxicity was measured in absence of O/V treatment (non attributable to CTX-like compounds), the content in CTX1B was quantitatively estimated by substituting the quantity of CTX1B responsible for the IC_{50} of the CTX1B calibration curve (with O/V treatment) for the number of *G. excentricus* cells also responsible for the IC_{50} in both experimental conditions (O/V treated and non-treated cells). The equivalent of CTX1B in *G. excentricus* cells was finally estimated after subtraction of the content of CTX1B equivalents estimated with O/V treatment with the content of CTX1B equivalents estimated without O/V treatment as described previously. (Caillaud et al., 2011; Caillaud et al., 2010a; Caillaud et al., 2010b; Lartigue et al., 2009).

When differences between IC_{50}s for SK&F 96365 treated and non treated cells were significant ($p < 0.05$), production of MTX-like compounds by *G. excentricus* was qualitatively determined by the measurement of a dose-ratio (DR) above 1 (Caillaud et al. 2010b). When DR>1, the content in MTX equivalents was quantitatively estimated by substituting the quantity of MTX responsible for the IC_{50} of the MTX calibration curve with SK&F 96365 treatment for the number of *G. excentricus* cells responsible for the IC_{50} of the microalgal extract with SK&F 96365 treatment (Caillaud et al., 2011; Caillaud et al., 2010b).

3. Results

3.1. ***Gambierdiscus excentricus*** **S. Fraga** ***sp. nov.***

Cellulae photosyntheticae quarum forma lenticularis est et mensurae mediae earum sunt: 97µm positione dorsiventrali, 83 µm latitudine et 37 µm longitudine. Thecae formula est: Po, 4’, 0a, 6’’, 6c, ?s, 5’’’, 0p, 2’’’’. Thecae laminae sunt laeves et poros rotundos et ovales uniformiter ordinatos habent. Lamina apicalis pori, Po, ovalis est, habet rimulam hamuli forma et ventraliter lapsa. Lamina prima apicalis 1’ parva est. Secunda apicalis lamina 2’ maior ex epitheca est et suturam 2’/3’ habet fere duplicer longiorem quam suturam 2’/4’. Placae 1’ et 6’’ parvissimae sunt et respiciunt ad posteriorem caellulae partem torsionis causa areae flagellaris, quae habet cavum ex quo dua flagella emergunt, quorum longitudinale perpendiculariter projicitur. Lamina S.p. locatur in hypotheca extra sulcum. Lamina 2’’’’ duplo longa est quam lata. Nucleus arcus formam habet et locatur in parte dorsale caellulae et cuspides ejus diriguntur ad ventralem partem. Toxica est et generat ciguatoxina atque maitotoxina.

Cells of *G. excentricus* are lenticular in shape with average depth 97 ± 8 (84-115) µm, width 83 ± 10 (69-110) µm, and length 37 ± 3 (34-41) µm. Thecal plate formula: Po, 4’, 0a, 6’’, 6c, ?s, 5’’’, 0p, 2’’’’. Thecal plates are smooth with evenly distributed round to oval pores. Apical pore plate Po is oval with a fishhook-shaped slit and is ventrally displaced. First apical plate, 1’ is very small. Second apical plate 2’ is the largest of the epitheca and has the suture 2’/3’ about twice as long as the suture 2’/4’. Plates 1’ and 6’’ are very small and facing the posterior part of the cell due to the torsion of the flagellar area which forms a hollow from which two flagella emerge, the longitudinal one being perpendicularly projected. S.p. is situated out of the sulcus in the hypotheca. 2’’’’ is about twice as long as wide. The nucleus is arc shaped and is located in the dorsal part of the cell with points towards the ventral side of the cell. Cells are photosynthetic.

Holotype: Fig. 2 from clonal strain VGO790, barcoded in GenBank (GenBank ID: JF303074) , (GenBank ID: HQ877874) and (GenBank ID: JF303065) and with preserved DNA at Centro Oceanográfico de Vigo (IEO). Clone VGO790 was collected on March 28th, 2004 as an epiphyte on small filamentous macroalgae and turf on a tidal pond in Punta Hidalgo, Tenerife Island, Spain (Fig. 1). It is deposited at the Culture Collection of Harmful Microalgae of Centro Oceanográfico de Vigo (CCVIEO).

Etymology: Refers to the position of the Po plate which is ventrally displaced compared to other species of *Gambierdiscus* in which it is centrally located.

Type locality: Punta Hidalgo, Tenerife Island (Spain) (28º 34' 37”N; 16º 19' 42”W) (Fig. 1).

Distribution: G. excentricus is only known from the Canary Islands of Tenerife, La Gomera and La Palma.

3.2. Morphology

Armored lenticular cells, anteroposteriorly compressed, with average depth (dorso-ventral axis) 97 ± 8 (84-115) µm, width 83 ± 10 (69-110) µm, and length (Antero - posterior axis) 37 ± 3 (34-41) µm. In apical or antapical view the cell is oval and indented in the ventral area showing a lobe in the right side (Figs. 2, 3, 4). In recently divided cells this lobe is more prominent in one of the two daughter cells (Fig. 5). Young cells are oval in apical view, but the dorsal side of old cells is flat (Fig. 5b). Epitheca and hypotheca are similar in height, smooth and covered by evenly distributed round pores of about 0.5 µm in diameter and at a concentration of 54 ± 10 per 100 µm^{-2} (Fig. 4). The plate formula is Po, 4', 0a, 6", 6c, ?s, 5"', 0p, 2"". Po is ventrally displaced and has a fishhook-shaped slit surrounded by a row or pores (Fig. 4d). It contacts three apical plates: 2’, 3’ and 4’ which overlapped it (Fig. 2). Plate 1’ is very small and arrow point shaped; it does not contact Po but contacts 4’ with the anterior point and is compressed by 1’’ and 6’’ forming like a groove with small wings having a cingulum-like appearance (Fig. 4c). The tiny 1’ and 6’’ are orientated towards the posterior side of the cell so they are not visible in apical view and only the lists bordering 1’ are visible in this view. Plate 2' is more or less rectangular and is the biggest of the epitheca; it is dorsally pointed and it is overlapped by 3’, 4’, 1’’, 2’’ and 3’’; as a result of the ventrally displacement of Po, its 2'/3' suture length is more than twice as long as 2'/4' suture length (Figs. 2a, 3a, 4a, 6). Plate 3’ is dorsoventrally elongated and overlaps 2’, 4’ while it is overlapped by 3’’, 4’’ and 5’’. Plate 4’ is smaller than 2’ and 3’, and in the ventral end overlaps the tiny 1’ and 6’’ plates. Plate 1’’ is five sided and overlaps contacts 1’, 4’, and 2’, and it is overlapped by 2’’. Plate 2" is four sided and together with 3", which is five sided, they are the biggest of the precingular series and occupy the whole dorsal part of that series. Plate 2’’ overlaps 1’’ and 2’, and is overlapped by 3’’. Plates 1’’, 4’’ and 5’’ have an intermediate size and plate 6" is very small. The cingulum is descendent one girdle width but, in ventral view the flagellar area appears twisted clockwise giving the appearance of being ascendant (Fig. 4c). It is composed of 6 plates being c1 and c6 curved due to the torsion of the flagellar area. The sulcus forms a hollow and S.p. is out of it forming part of the hypotheca. S.a. is in contact with 1' and 6''. The hollow is limited in the posterior side by the anterior edges of 5''', S.p., and 1'''. It was not possible to analyze all the sulcal plates. The longitudinal flagellum emerges in the equatorial plane perpendicularly from the hollow and below plate 5'' when observed in apical view (Fig. 2a). The transverse flagellum finished well inside the hollow.

The hypotheca is composed by five postcingular plates and two antapical plates in addition to S.p. which being out of the sulcus is considered as 2'''' by many authors. 1''' is triangular and is the smallest of the series, 2''' trapezoidal being the dorsal part wider than the ventral part. Plate 3''' is four sided and dorsally placed. Plate 4''' is elongated and occupies most of the right side of the postcingular area being the biggest of the postcingular series. Plate 5''' is small and twisted. In the antapical series, 1'''' is more or less symmetrical to S.p. and contacts 1''', 2''', 2'''' and S.p. (Figs. 2b, 3b, 4c). Plate 2'''' contacts five plates, 1'''', 2''', 3''', 4''' and S.p. and it doesn't contact 5'''. The width of 2'''' is about one third of the transdiameter and is about twice as long as wide, being wider towards the ventral side (Figs. 2b, 3b, 4b). 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 (Figs. 2,7) After division, the daughter cell that bears the other side which includes the plates that form the ventral right lobe, 5'' and 5''', is very asymmetrical in apical view (Fig. 5c), while the other cell appear more symmetrical with both lobes almost the same size. (Fig. 5d). *G. excentricus* has numerous and small chloroplasts radially dispersed. The nucleus forms an arc in the dorsal side with points towards the ventral side (Fig. 8).

In a different sample from the Canary Islands, *Gambierdiscus* cf. *polynesiensis* was found and was isolated as strain VGO1022. It is smaller in size, has a centrally located Po and a wide 2’’’’. It will be the subject of a future study.

3.3. Ecology and behavior

G. excentricus was found in tidal ponds on rocky shores of volcanic origin in areas very exposed to the intense trade winds of Tenerife, La Palma and Gran Canaria Islands (Fig 1). The cells were on small macroalgae and turf although they were found also in drifting small seaweeds in a protected rocky inlet in La Gomera Island leeward of trade winds. Sea surface temperature in the area ranges from about 18 ºC to 24 ºC and salinity ranges from 36.6 to 36.8 during winter and some years can reach 37 in summer (Neuer et al., 2007). Nevertheless, sampling cannot be considered as representative as it was done opportunistically. In comparison to other *Gambierdiscus* species in culture, *G. excentricus* cultured in our laboratory is a very sedentary species as it is almost non motile, and usually the only appreciable movements are the beaten of longitudinal flagellum and the undulating movement of the transverse flagellum. *G. excentricus* cells were not observed swimming and the two daughter cells usually appear close one to one another after division. In our culture conditions cells appeared more concentrated in the more illuminated areas of the flasks.

3.4. Genetics

The phylogenetic results for D1-D3 and D8-D10 LSU sequences are shown in Figs. 9 and 10. Both trees displayed a similar topology confirming that *G. excentricus* sequences clustered into a well supported group, separated from the rest of *Gambierdiscus* species and with *G. australes* as its closest relative. To inspect the differences between *G. excentricus* and the other studied species we calculated the uncorrected genetic distance (p) between the consensus sequences of each species/clade included in the phylogenetic analyses. The minimum number of substitutions per site was obtained for the pair *G. caribaeus*/*G. carpenteri* (0.067 and 0.006 in D1-D3 and D8-D10 original alignments) and *G. yasumotoi*/*G. ruetzleri* (0.009 and 0.008). *G. excentricus* had significantly larger p values (0.350 and 0.083) relative to *G. australes*, its sister group in the analyses. The distance between *G. excentricus* and *G. australes* is also larger than that calculated between *G. toxicus* vs *G. belizeanus* (0.181-0.242 and 0.054 in D1-D3 and D8-D10 original alignments). The D8-D10 sequence of strain VGO1022 was placed in a separate clade which included two sequences from *Gambierdiscus* “ribotype I”, as defined by Litaker et al. (2010). However, the similarity observed between strain VGO1022 and other G. *polynesiensis* sequences in the D1-D3 phylogeny (Fig. 9) indicates that *Gambierdiscus* ribotype I probably belongs to *G. polynesiensis*. Additional work should be carried out to confirm its actual taxonomical status.

3.5. Pigment composition

The HPLC chromatogram (Fig. 11) shows the standard peridinin (Per)-containing chloroplast with chl c_2 and Per as major accessory pigments. Chl c_1 was a minor pigment (chl c_1/chl c_2= 0.13) previously detected in the genus *Gambierdiscus* (Durand and Berkaloff, 1985). Diadinoxanthin (Diadino) and dinoxanthin (Dino) are also relevant pigments with different contribution to the carotenoid pool. Pigment ratios respect to chl *a* for carotenoids vary from Per/chl *a* = 1.56, Diadino/chl*a* = 0.41 to Dino/chl *a* = 0.14 while chl c_2/chl *a* = 0.46.

3.6. Toxicity.

All the strains of *G. excentricus* were toxic to Neuro-2a cells with and without O/V treatment (Table 2). Toxic effects were significantly higher in the presence of O/V treatment ($p < 0.05$) thus indicating the production of CTX-like compounds by the three strains of *G. excentricus* studied.

All the strains of *G. excentricus* were also toxic to Neuro-2a cells with and without SK&F 96365 treatment (Table 2), with toxic effects significantly different between both treatments ($p < 0.05$). DRs calculated for *G. excentricus* strains were above 1, suggesting the production of MTX-like compounds by the three strains studied.

Estimations of the equivalents of CTX1B and MTX per cells produced by the three strains of *G. excentricus* are given in Table 1. Strains VGO790 and VGO791 produce significantly higher contents of CTX1B equivalent per cells respect to VGO792

(ANOVA, p<0.01). Production of MTX equivalents per cells by strain VGO790 was significantly higher than strains VGO791 and VGO792 (ANOVA, p<0.001). Additional work with higher amounts of *G. excentricus* biomass obtained from larger scale cultures will be necessary to improve extraction and separation of MTX from CTX to confirm the amounts of toxins produced.

4. Discussion

4.1. Morphology.

As already noticed previously by Litaker et al. (2009), a discrepancy exists among different authors on the nomenclatures to describe the tabulation of *Gambierdiscus*. When (Kofoid, 1909) proposed his famous nomenclature system for the plates of dinoflagellates, he chose the names of apical, precingular, postcingular and antapical and intercalary plates in order to have a common criterion of nomenclature. When comparing different genera or species, it is possible to find that homologous plates in different species need to be called with different names if the Kofoid criterion is strictly used. This obviously does not help comparisons. This problem was discussed in the Penrose Conference on “Modern and Living dinoflagellates” held in Colorado Springs, USA in 1978 and several publications followed (Balech, 1980; Eaton, 1980; Edwards, 1990; Taylor, 1979b), which include proposals of new nomenclatural systems that should facilitate the study of homologous plates. A detailed discussion is in (Fensome et al., 1993). Although the new systems facilitate these studies, the modified

Kofoid system is still in use and none of the new systems succeeded among conservative neontologists. One of the problems of the Kofoid system concerns the first apical plate (1’), called “1s” or “1u” in the Taylor-Evitt system (Evitt, 1985; Fensome et al., 1993; Taylor, 1979b, 1980) which in some *Alexandrium* species should be called first precingular plate instead of first apical, because it doesn’t touch Po. In this case, the formula of the epitheca should be different for different species of the same genus. Due to the toxic character of many of the species of *Alexandrium*, many papers on this genus have been published, and in them it became normally accepted that the first apical plate can be disconnected from Po and is still being called “apical” by most of the authors. In this paper we applied for *Gambierdiscus* a modified Kofoidian nomenclature system as used for *Alexandrium* by Balech (1995) for this genus and by Besada et al., (1982) for *Gambierdiscus, Ostreopsis* and *Coolia*. Gonyaulacales can be grouped in three types according to the plates that contact the homologous to 1’ plate (Fensome et al., 1993). If 1’ contacts Po, the type is ‘insert’, if this contact is interrupted by 2’ and 4’, it is ‘metasert’ and in the case that the contact between 1’ and 2’ is interrupted by 1’’ it is ‘exsert’. In genus *Alexandrium* the three different types can be found: *A. tamarense* is insert, *A. monilatum* is metasert and *A. margalefi* is exsert, so there is no reason to give these plate different names. Plate 1’ in *G. excentricus* is minute and does not contact Po being of the exert group of species of Gonyaulacales as *A. margalefi.* Similar arguments can be applied to sulcal posterior plate (S.p.) of *Alexandrium,* named “Z” in the Taylor-Evitt system. Its homologous plate is out of the sulcus in *Gambierdiscus, Coolia* and *Ostreopsis* (Besada et al., 1982; Taylor, 1979a) as in *Goniodoma sphaericum* (Balech, 1980). On doing this, the plate formula for these Gonyaulacacean genera is the same as follows: Po, 4’, 0a, 6’’, 6c, ?s, 5’’, 0p, 2’’, and allows comparisons among them. Plate 1’’’’ has a wing in the side contacting the sulcus as it happens in *Alexandrium* and *Coolia,* but as in *Gambierdiscus* the ventral area is clockwise twisted, this wing, instead of being faced towards the right side of the cell, is facing the ventral or anterior side. For the same reason, S.p. is displaced to the right side of the hypotheca instead of being central as in *Coolia* and most *Alexandrium* species. Plate 2'''' contacts five plates, 1'''', 2''', 3''', 4''' and S.p. and, like genus *Goniodoma* and *Alexandrium*, and unlike *Coolia* and *Ostreopsis*, it does not contact 5''' (Fensome et al., 1993).

The main character used to differentiate *G. excentricus* from other species of lenticular *Gambierdiscus* is the high ratio between the 2'/3' and 2'/4' suture lengths. Such a morphological character is unique among all the discoid known species of genus *Gambierdiscus* . While in *G. excentricus* this ratio is around 2.3, in the other discoid species ranges between 1.0 and 1.6 . The shape of plate 2' is one of the characteristics used to differentiate species of *Gambierdiscus* (Litaker et al., 2009) and in all the described species the position of Po is more or less centered in the right side of 2', while *G. excentricus* is the only one among the discoid species having it ventrally displaced as in the globular species. This displacement makes that the contact of Po with 2' is also ventrally displaced and then, plate 2' has a peculiar shape (Fig. 6). In a SEM picture of a *Gambierdiscus* cell from the Moroccan coast, not far from the Canary Islands, this characteristic shape of plate 2' was also observed (B. Ennaffah pers. com.). Far from the NE Atlantic, this character was observed in figure 8 of Loeblich III and Indelicato (Loeblich III and Indelicato, 1986) but not in the other figures of the single clone studied by those authors in which this ratio is about 1.5, as the other discoid *Gambierdiscus* species. To explain these morphological differences among cells of the single clone these authors used (F-8), there are two possibilities: one is that their figure 8 shows an aberrant cell as many of the cells shown in other figures, and the other is that more than a clone could exist in that strain corresponding to different species, and one of these being *G. excentricus*. In a sample from Brazil a cell showing this characteristic 2’ plate was also observed (S. Nascimento pers. com.), which possibly could be *G. excentricus.* A high parallelism in the *Ostreopsis* cf. ovata populations between Brazil and the Canary Islands was observed (Penna et al., 2010), so, a similar distribution of another benthic species could be expected. Concerning the hypotheca, *G. excentricus* is in the group of species having a narrow 2’’’’ (1p for other authors) like *G. belizeanus*, *G. australes* and *G. pacificus* (Faust, 1995; Litaker et al., 2009; Richlen et al., 2008) from which it is easily distinguished in base of the shape of 2’.

Based on morphology, it can be discounted that the *Gambierdiscus* reported as *Goniodoma* by Silva (1956) in a sample from Cabo Verde, south of the Canary Islands, is *G. excentricus.* Nevertheless it cannot be discounted that Silva’s description of Goniodoma could be the same species as strain VGO1022 and close to *G. polynesiensis*.

4.2. Phylogeny

LSU generated a robust phylogeny delineating *G. excentricus* as a different specific clade. Both LSU trees were elaborated using selected sequences from two recent comprehensive studies on the genus *Gambierdiscus* (Litaker et al., 2009; Litaker et al., 2010). These authors noted that SSU phylogeny was more informative than LSU for discriminating species at deeper branches, although the resulting topologies were very similar. However, the LSU separation of *G. excentricus* from other related species (e.g. *G. australes*) is solid enough to discount further genetic verification. In a recent study, Litaker et al. 2007 screened the ITS/5.8S variation in 14 genera of dinoflagellates and proposed that uncorrected genetic distance (p) values exceeding 0.04 would represent the boundary at species-level. Based on this approach, Litaker et al. (2009) observed that very closely related *Gambierdiscus* species, as *G. yasumotoi*/*G. ruetzleri*, also fulfilled this rule. Given the higher genetic distance calculated on the basis of LSU phylogenies between *G. excentricus*/*G. australes* in comparison with *G. yasumotoi*/*G. ruetzleri* (see results), it would be also expected that *G. excentricus* displayed p values > 0.04 relative to *G. australes* in a ITS/5.8S alignment. Finally, in certain cases such as for the VGO791 strain, aberrant D1/D3 amplicons were obtained probably corresponding to pseudogene copies of the LSU, as previously noticed in *Gambierdiscus* and other dinoflagellates (Richlen and Barber, 2005; Litaker et al 2009). The D8-D10 sequence from strain VGO1022 matched the two *Gambierdiscus* ribotype I sequences selected in this study (Litaker et al., 2010), not retrieved from cultures until date. These authors suggested that *Gambierdiscus* ribotype I probably represented a new species based on the genetic distances found in D8-D10 region. However, the similarity observed between strain VGO1022 and other *G. polynesiensis* sequences in the D1-D3 phylogeny (Fig. 9) indicates that additional work should be carried out to confirm its actual taxonomical status.

4.3. Pigments

Peridinin-containing dinoflagellates contain chl c_2 and usually lack chl c_1 (Jeffrey et al., 1975). Only a few dinoflagellate species are exceptions for such a general statement. Chlorophyll c_1 was first detected in *Gambierdiscus* by Durand and Berkaloff (1985) when the separation of chl c_1 and chl c_2 was a methodological challenge. A further study of the pigment composition of *Gambierdiscus* by Indelicato and Watson (1986) described the detailed carotenoid composition; however, they failed to detect chl c_1. The identification of chl c_1 was verified by Bomber et al (1990) using proton nuclear magnetic resonance spectrometry (H^1-NMR). At present the HPLC methods are more selective toward chl *c* separation. All the *Gambierdiscus* strains here studied contained the same pigment pattern with slight difference in quantitative proportions expressed as pigment to chl *a* ratios. Although the occurrence of chl c_1 was suppressive in peridinin – containing dinoflagellates the simultaneous occurrence of both pigment is not a pigment signature due to other dinoflagellates share this trait.

4.4. Toxicity

G. excentricus was identified as a CTX and MTX producer according to the results of the Neuro-2a CBA. The content of CTX1B equivalent per cells quantified for strains VGO790, VGO791 and VGO792 (Table 1) was of the same order as previously reported for other species of *Gambierdiscus* spp. (Caillaud et al., 2010c; Rhodes et al., 2010). As an example, (Chinain et al., 2010) reported toxicity values according to Receptor Binding Assay (RBA) for *G. toxicus*, *G. australes*, *G. pacificus*, *G. belizeanus* and *G. polynesiensis* from French Polynesia ranging from 0.017 to 11.9 pg CTX3eq cell^{-1} (equivalent to 0.0017 and 1.19 pg CTX1B eq cell^{-1}), *G. polynesiensis* being described as a potent CTXs producer. *G. excentricus* strain production from the Canary Islands ranges between 0.37 and 1.1 pg CTX1B eq cell^{-1}. Regarding the production of MTX, poor data are available in the bibliography regarding the content of MTX produced by *Gambierdiscus* spp. Caillaud et al. (2010c) reported the production of 36.7

nmoles MTX·10^{-6} cells of *Gambierdiscus* sp from Indonesia, which is equivalent to 0.11 ±0.04 ng MTX $cell^{-1}$, *G. excentricus* strains from the Canary Islands produce between 0.48 and 1.38 ng MTX $cell^{-1}$ suggesting *G. excentricus* strain VGO790 as a potent MTX producer in relation to the Indonesian strains. However this observation would require the comparison of the MTX production by *G. excentricus* with a higher number of strains/species of *Gambierdiscus* spp. As previously described in the introduction of the present study, the production of MTX by *Gambierdiscus* spp may not threaten human health (Lewis, 2006). However presence of CTX-producing *Gambierdiscus* spp in a given ecosystem supposes a risk of CFP.

The first ever reported case of CFP in the Canary Islands, Spain (Fig. 1) was caused after consumption of local amberjack (*Seriola rivoliana*) in 2004 (Pérez-Arellano et al., 2005). The *in vitro* Neuroblastoma (Neuro-2a) cell-based assay (CBA) identified CTX-like toxicity and liquid chromatography with mass spectrometry detection (LC-MS) confirmed the presence of Caribbean type 1 CTX (C-CTX-1) together with two other unidentified toxins (Pérez-Arellano et al., 2005). The same year of the intoxications and in an independent study, *Gambierdiscus* sp. was found in the Canarian coast (Aligizaki et al., 2008). More cases were reported in the Canary Islands in 2008 and 2009 caused in both cases by amberjacks that were captured near Selvagem Islands, Portugal (Fig. 1), at 175 km north of Canary Islands, and as in the 2004 case, C-CTX1 was detected by LC-MS/MS (Boada et al., 2010). In these islands several cases of ciguatera were reported but no analyses were done on the meals of the affected people (Gouveia et al., 2009), but several ciguatoxins in addition to C-CTX1 were detected later by LC-MS/MS in amberjacks captured in the area (Otero et al., 2010). Although the presence of *Gambierdiscus* and the cases of ciguatera in the East Atlantic were only recently reported, this was probably due to lack of studies. *Gambierdiscus* sp.

was observed in the area as early as 1948 in the Cabo Verde archipelago, although reported as *Goniodoma* sp. (Silva, 1956) and it can be considered as the first record of this genus. Comparing the figure of Silva (1956) with *G. excentricus* we can conclude that they correspond to different species of *Gambierdiscus* but, nevertheless the species of Cabo Verde could be the same as the second species found in Canaries but more studies are necessary. The first historic record of ciguatera in the world could be also from the Eastern Atlantic. In 1525, at the beginning of the second circumnavigation of the world, a fleet of seven Spanish ships anchored in the island of San Mateo, which probably corresponds to which today is known as Annobon, in the Gulf of Guinea. The direct translation from the original report in Spanish says: “On this island, a very beautiful fish was caught in the flagship, called barracuda, and the Captain General invited some of the captains and officers of the King. All who ate the barracuda fell ill from diarrhea and were unconscious, so we thought they had died; however our Creator wanted everyone to be saved.” (Urdaneta, 1580). This incident was considered very important in its time as it was described with similar words in other reports of the same travel. As all the captains who were poisoned, died during this cruise few months later of unknown causes different from the common and well known scurvy, ciguatera is considered as a probable cause of their dead (de Miguel, 2009). Among the dead, was Juan Sebastián Elcano who was the first captain to circumnavigate the world only few years before. The recent identification of ciguatoxins in fishes of Cameroon (Bienfang et al., 2008), very near of the Island of Annobon, and the fact that the intoxications were caused by a big barracuda, support the consideration of these poisonings to be the first record of an outbreak of ciguatera in the world.

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FIGURE LEGENDS

Figure 1. (a) Map of the East Atlantic archipelagos. (b) Map of the Canary Islands showing the localities where *Gambierdiscus excentricus* was found.

Figure 2. Ink drawings of *Gambierdiscus excentricus*. (a) Apical view. (b) Antapical view. Gray arrows indicate direction of plates overlap. (c) Ventral view. Dotted lines show fission line.

Figure 3. Confocal microscope image of *Gambierdiscus excentricus* after calcofluor staining. (a) Apical view. (b) Antapical view. Scale bar. 20 µm.

Figure 4. SEM images of *Gambierdiscus excentricus*. (a) Apical view. (b) Antapical view. (c). Sulcal area. (d) Po plate. Scale bars: (a, b) 20 µm, (c) 10 µm, (d) 5 µm.

Figure 5. Morphological differences on descendants of a single cell of *Gambierdiscus excentricus* observed in antapical view. (a) Empty theca after ecdysis. (b) Same cell after 8 days growing. (c, d) Daughter cells after division three days later. (e - h) Third generation of cells. Scale bar. 20 µm.

Figure 6. LM figures of eight plates 2’ of *Gambierdiscus excentricus*.

Figure 7. Calcofluor stained epithecas and hypothecas of cells of *Gambierdiscus excentricus* recently divided in which the different intensity of staining permits the identification of fission lines. Scale bar. 20 µm.

Figure 8. *Gambierdiscus excentricus*. (a) Epifluorescence image of the U-shaped nucleus in apical view stained with SybrGreen. (b) Epifluorescence image of chloroplasts. Scale bar: 20 µm.

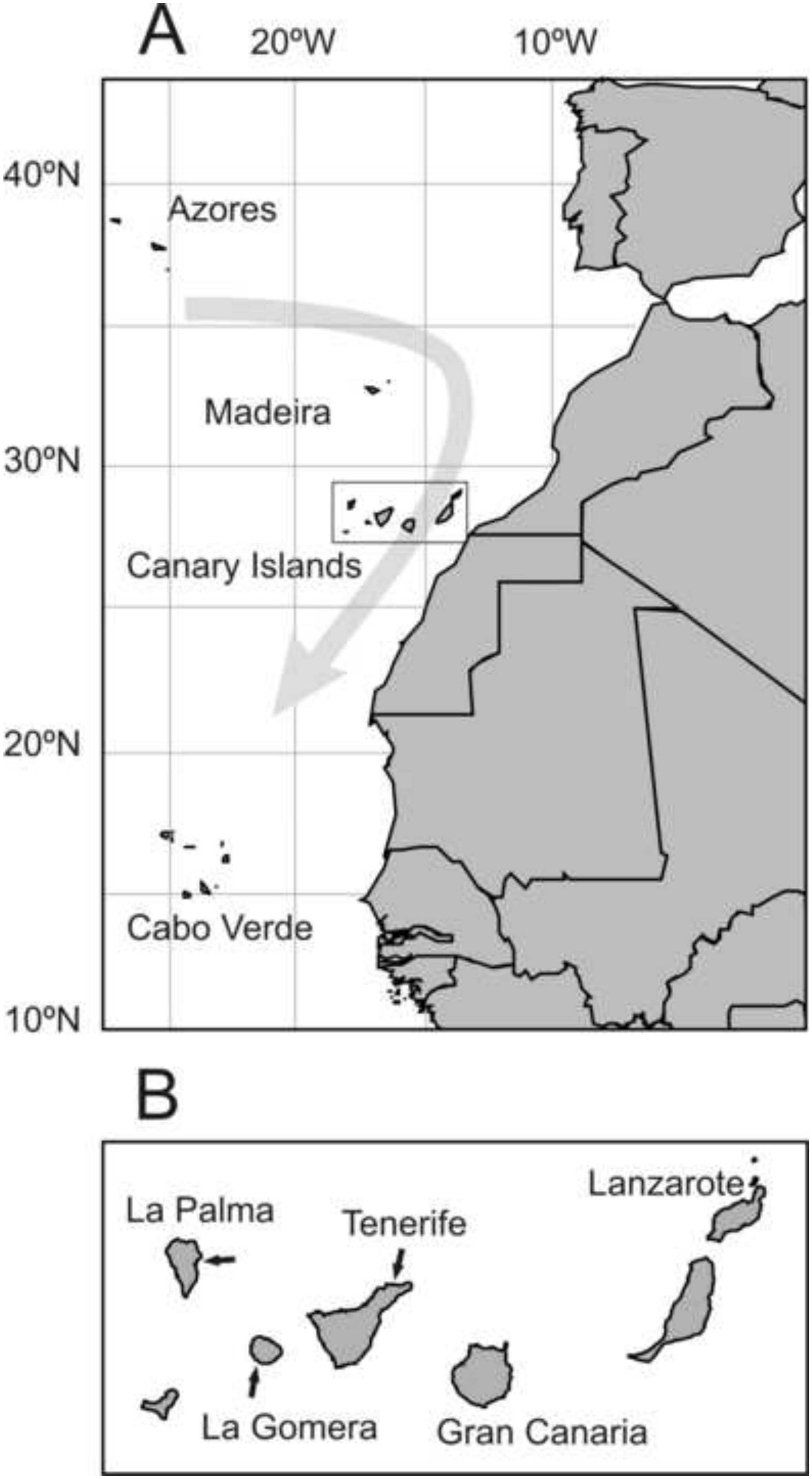
Figure 9. LSU phylogeny (D1-D3 region) showing the relationship between *Gambierdiscus excentricus* and other *Gambierdiscus* species. The additional numbers that follow each isolate obtained in this study refer to different LSU copies that were PCR amplified and sequenced among descendants from single cells of that isolate. Supports at internal nodes are posterior probability values (Bayesian analyses) and bootstrap values obtained by Neighbor Joining and Maximum Parsimony methods. Hyphens indicate bootstrap values <60. The GenBank accession numbers for the isolates obtained in this study are as follows: *G. excentricus* VGO 790, (GenBank ID: HQ877874) and (GenBank ID: JF303065); VGO 791, (GenBank ID: JF303066-68); VGO 792, GenBank ID: JF303069-71); VGO 1035, (GenBank ID: JF303063), *G.* cf. *polynesiensis* VGO 1022, (GenBank ID: JF303064). Accession numbers from other *Gambierdiscus* sequences are detailed in (Litaker et al., 2009).

Figure 10. LSU phylogeny (D8-D10 region) showing the relationship between *Gambierdiscus excentricus* and other *Gambierdiscus* species. The additional numbers

that follow each isolate obtained in this study refer to different LSU copies that were PCR amplified and sequenced from single cells of that isolate. Supports at internal nodes are posterior probability values (Bayesian analyses) and bootstrap values obtained by Neighbor Joining and Maximum Parsimony methods. Hyphens indicate bootstrap values <60. The GenBank accession numbers for the isolates obtained in this study are as follows: *G. excentricus* VGO 790 (GenBank ID: JF303074); VGO 791, (GenBank ID: JF303075); VGO 792, (GenBank ID: JF303076); VGO 1035, (GenBank ID: JF303073), *G.* cf. *polynesiensis* (labeled VGO 1022), (GenBank ID: JF303077), *G. australes* (VGO 1046, JF303072). Accession numbers from other *Gambierdiscus* sequences are detailed in (Litaker et al., 2010)

Figure 11. HPLC chromatogram of *G. excentricus* strain VGO1035. Peak identification: (1) peridininol, (2) divinyl protochlorophyllide (MgDVP), (3) chl c_2, (4) chl c_1 (5) peridinin, (6) peridinin-like, (7) pyrrhoxanthin, (8) diadinochrome, (9) diadinoxanthin, (10) dinoxanthin, (11) diatoxanthin, (12) unknown carotenoid, (13) chl *a* allomers, (14) chl *a*, (15) β, β-carotene. Detection by absorbance at 440 nm.

Figure 1


A
20°W
10°W
40°N
30°N
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10°N
Azores
Madeira
Canary Islands
Cabo Verde
B
La Palma
Tenerife
Lanzarote
La Gomera
Gran Canaria

Figure 2

Click here to download high resolution image

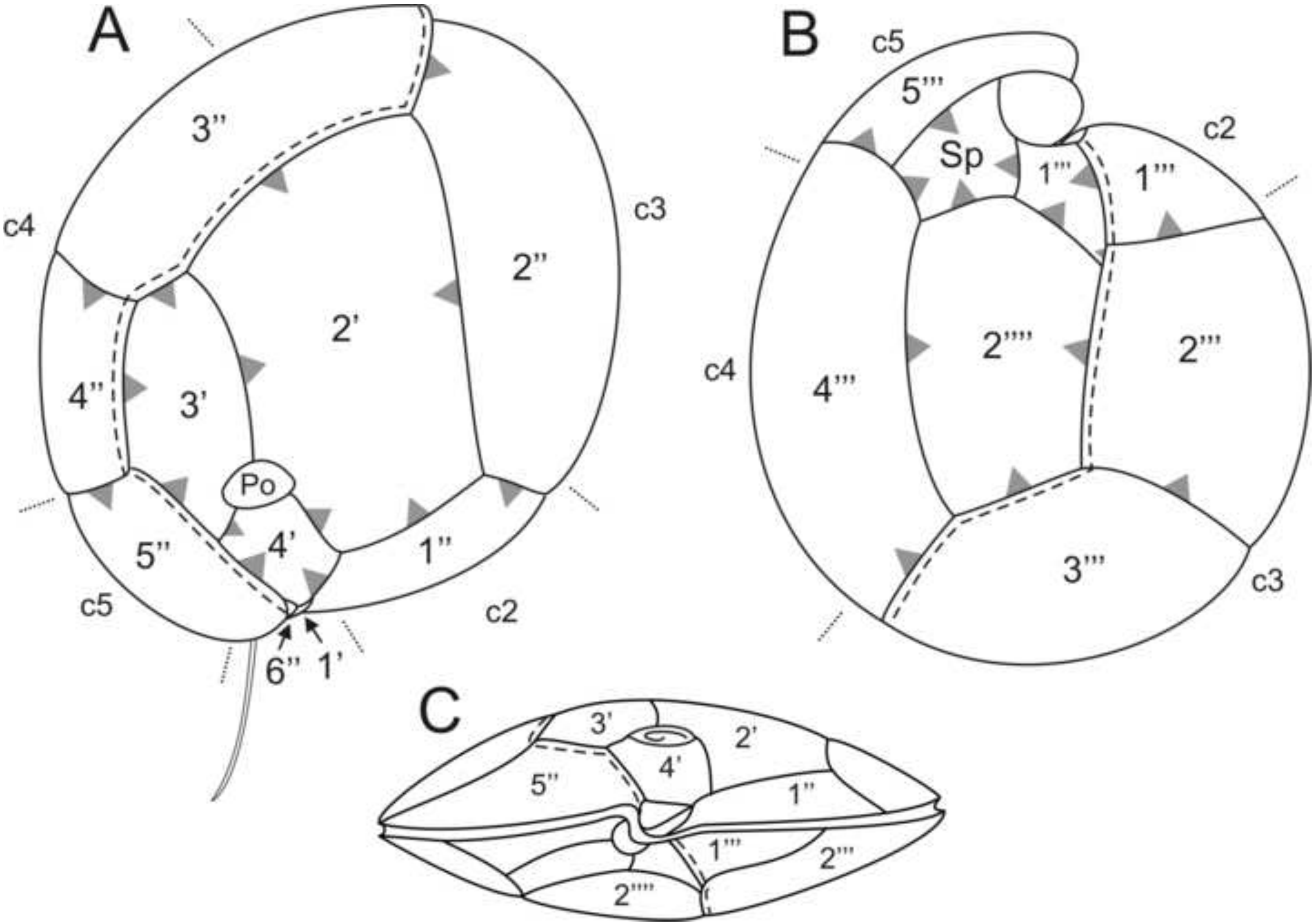

Figure 3

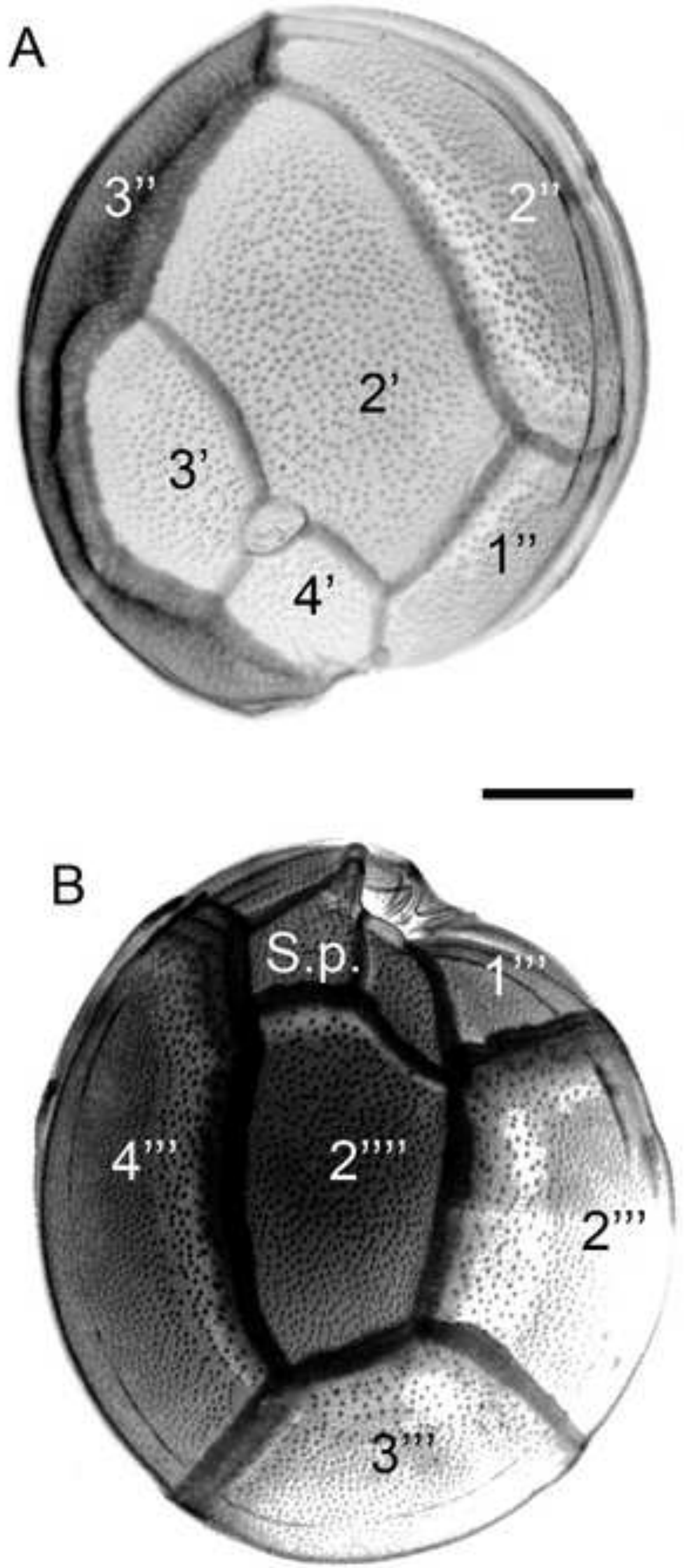

Figure 4

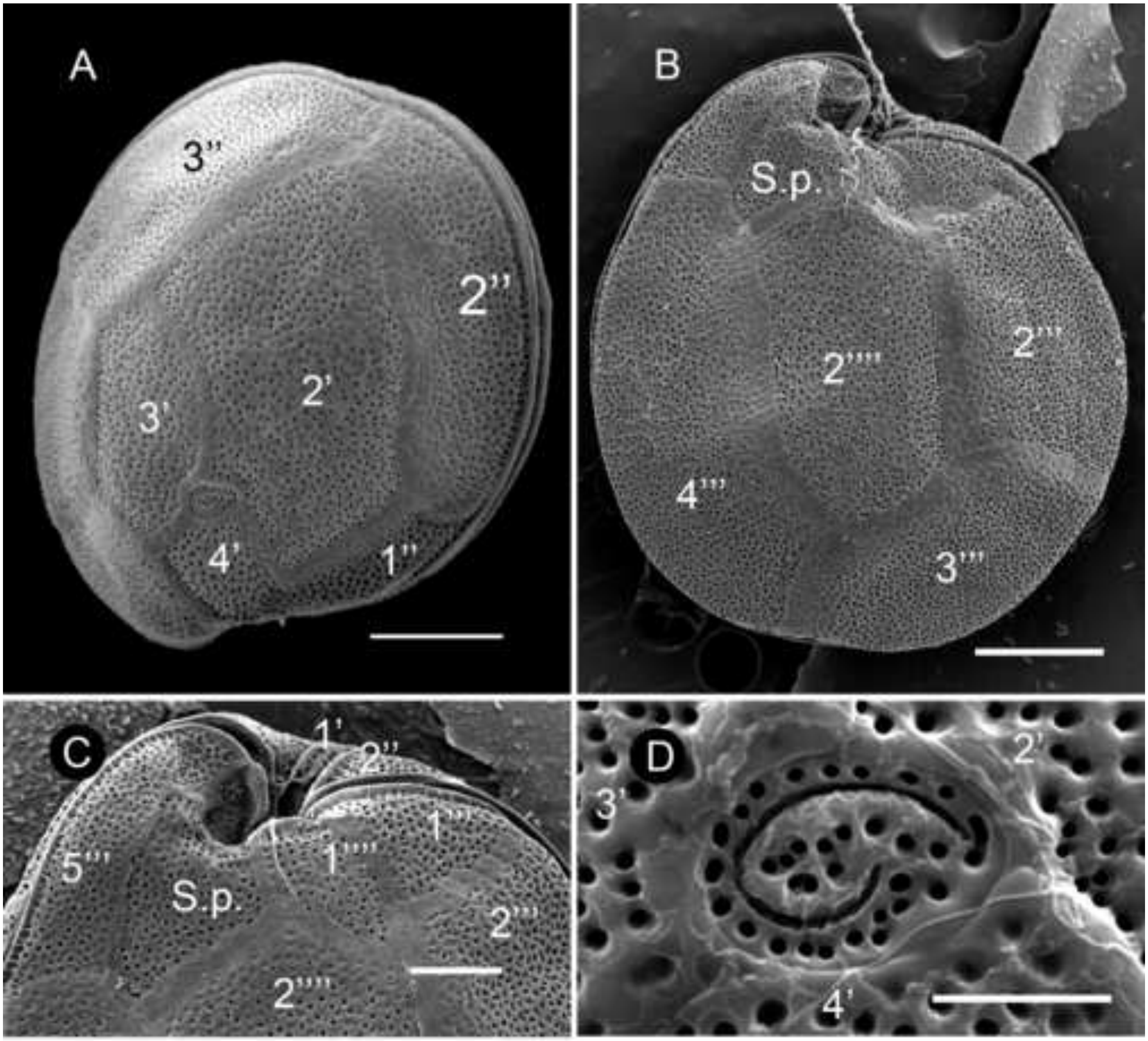

Figure 5

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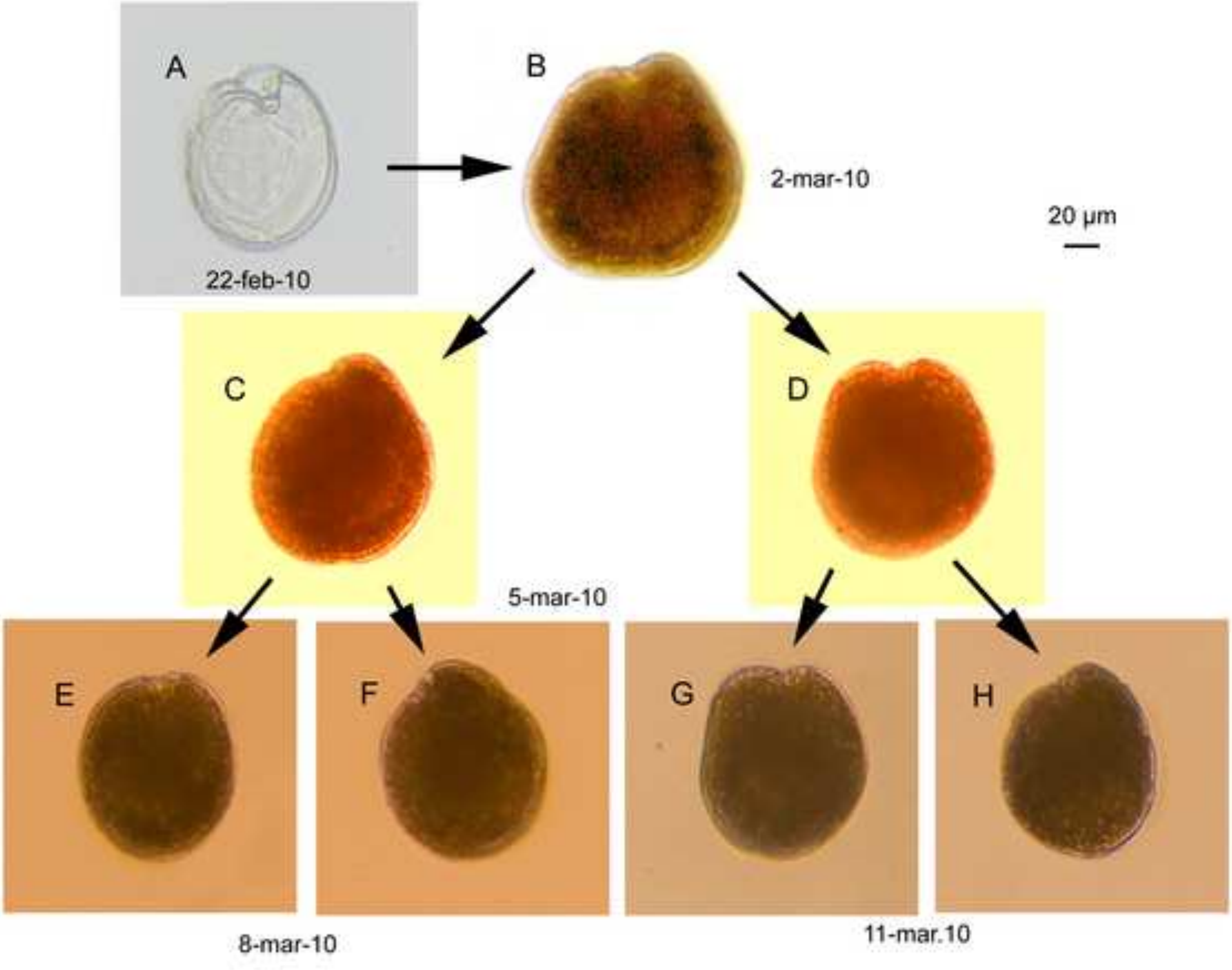

Figure 6

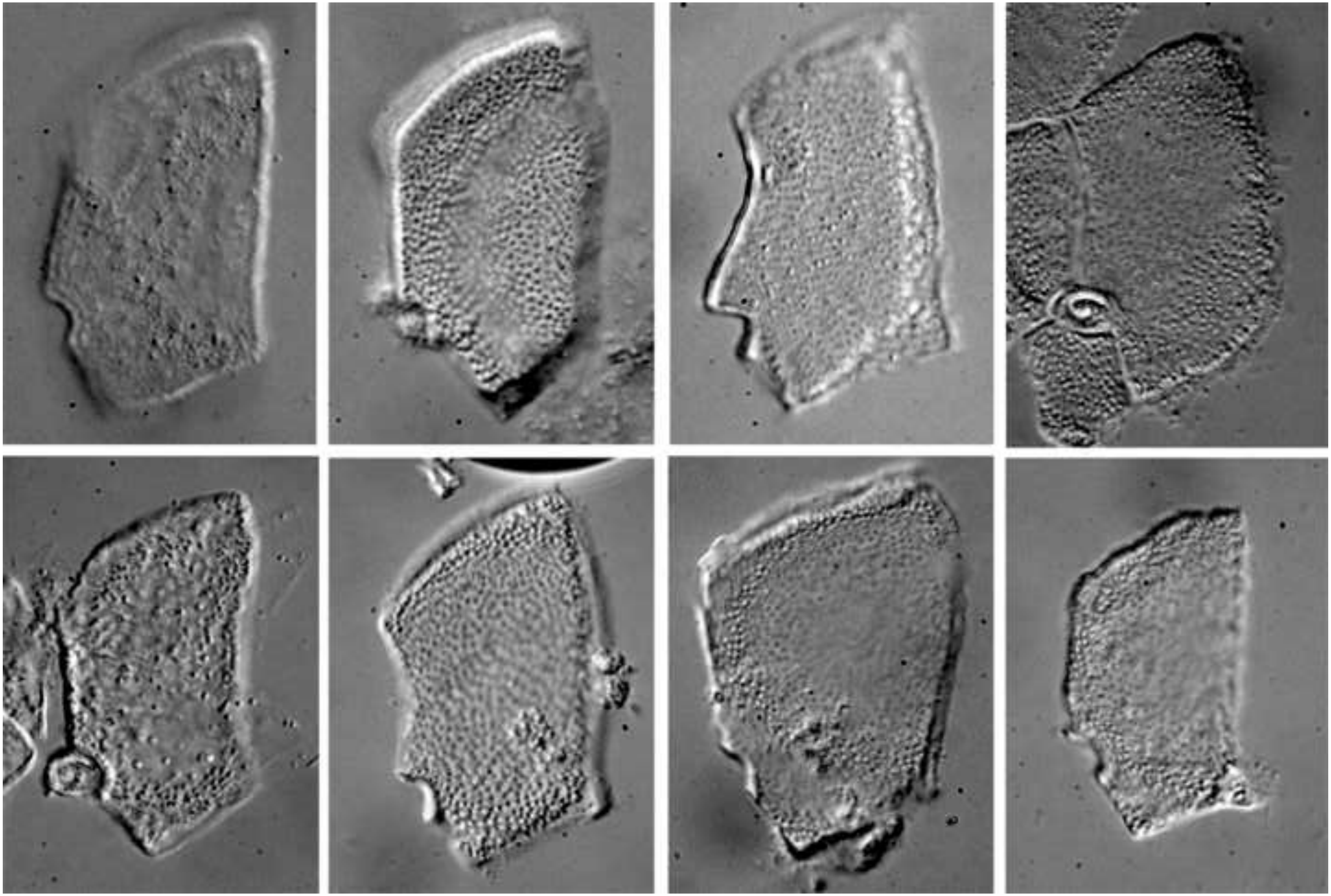

Figure 7

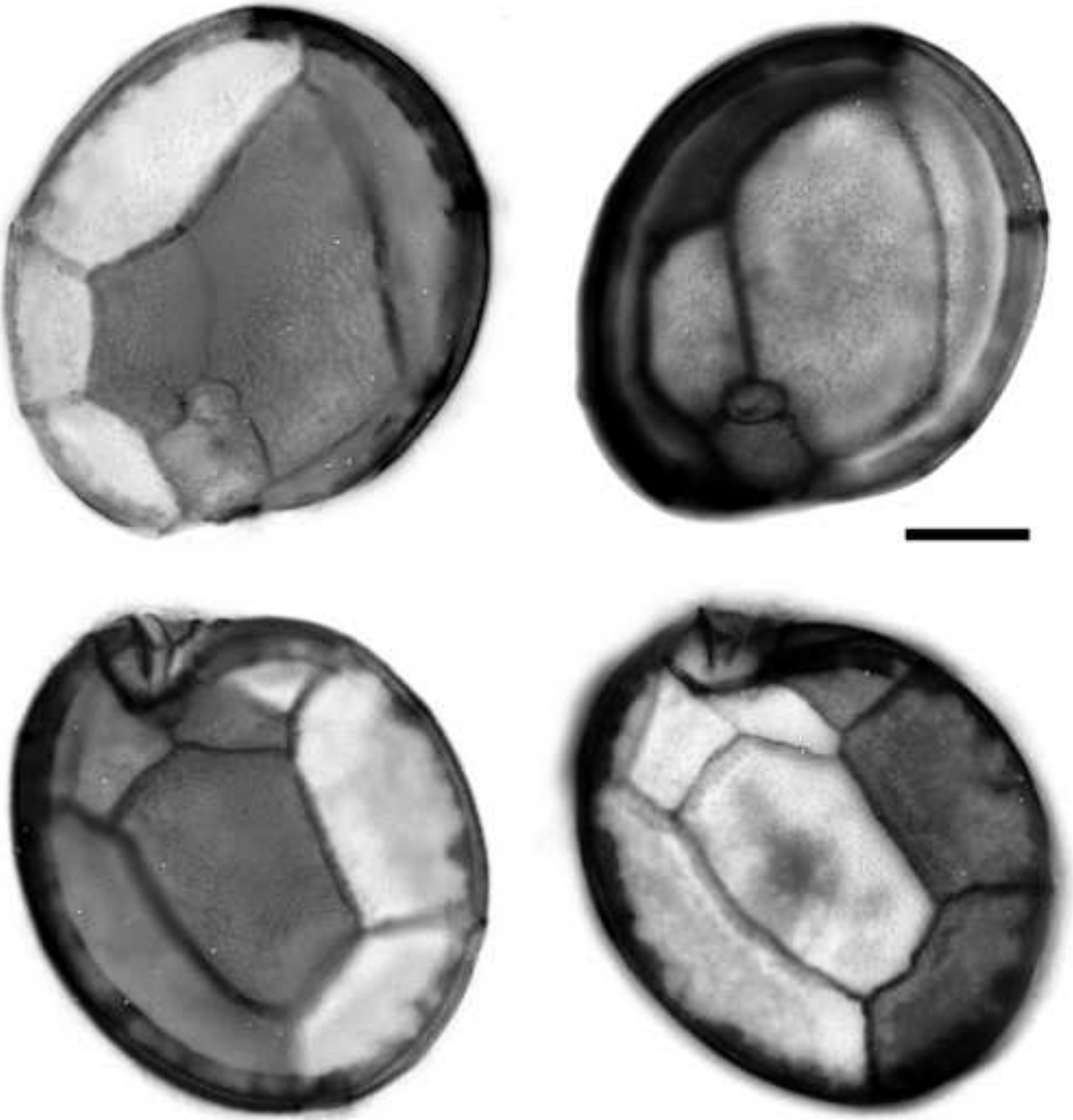

Figure 8

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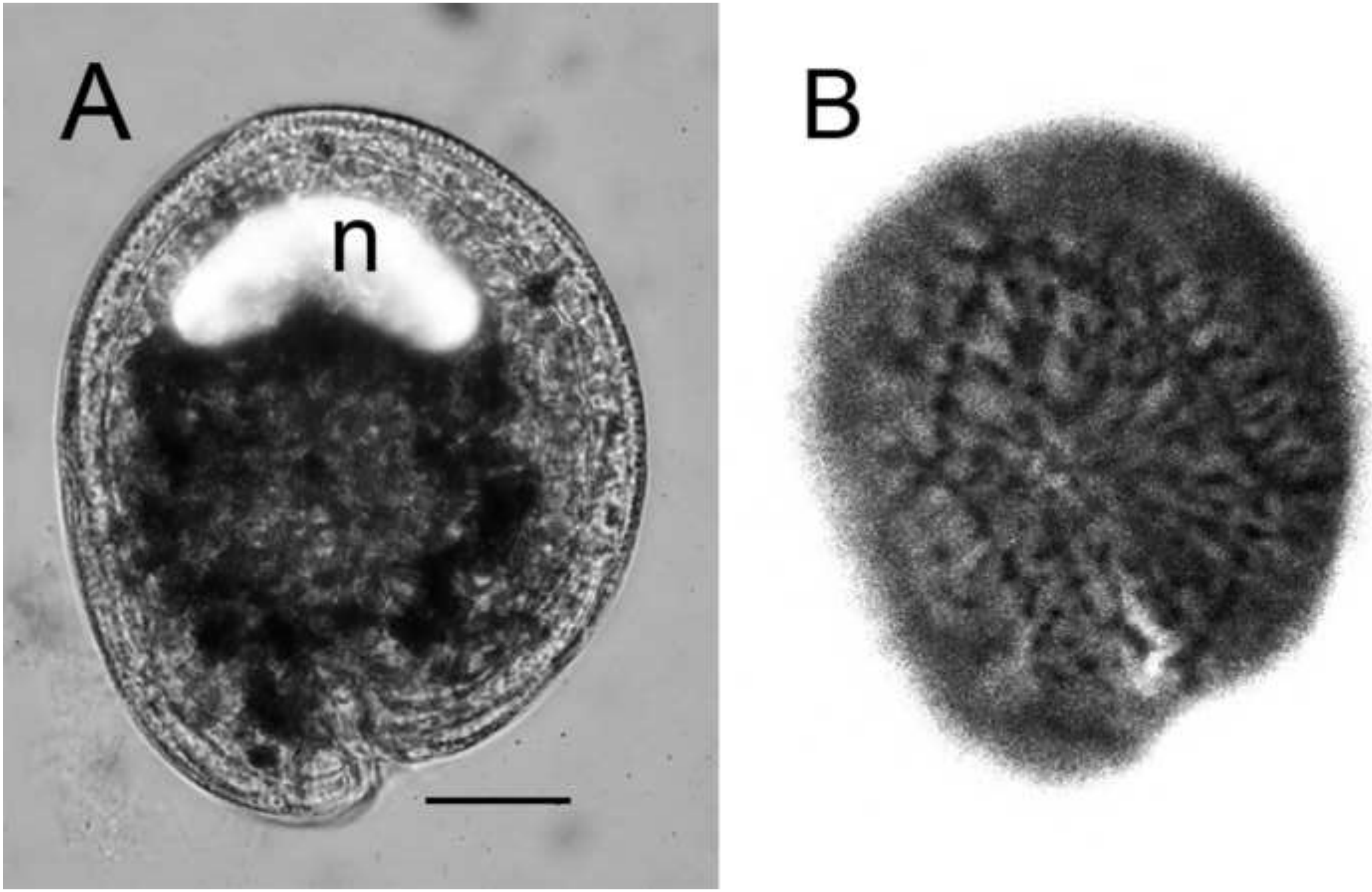

Figure 9


D1-D3
Gyasu 4
Gyasu 1
Gyasu 2
Gyasu 3
G. yasumotoi
99
NOAA8 1 1
NOAA8 1 3
NOAA8 1 6
G. ruetzleri
81
100
NOAA6 1 1
NOAA6 1 3
NOAA6 1 6
G. carolinianus
100
VGO 1022
100
RG 92-1
RG 92-4
TB 92-4
TB 92-3
RG 92-3
G. polynesiensis
100
NOAA5 1 8
NOAA5 1 2
NOAA5 1 3
G. belizeanus I
100
100
NOAA2 6
NOAA2 8
G. belizeanus II
100
60
100
HO91 1
HO91 2
HO91 3
HO91 4
G. pacificus
100
GTT91 1
GTT91 4
REN1 4
REN1 1
REN1 2
92
TUR1
TUR2
TUR3
TUR4
G. toxicus
55
VGO 1035
VGO 790 1
VGO 791 1
VGO 792 1
100
VGO 790 2
VGO 791 2
VGO 792 2
VGO 791 3
99
VGO 792 3
G. excentricus sp. nov.
87
87
100
RAV92 4
RAV92 1
RAV92 2
RAV92 3
G. australes
94
NOAA1 2
NOAA1 5
G. carpenteri
100
80
NOAA20 1 7
NOAA10 1 6
NOAA11 1 3
NOAA11 1 2
100
NOAA11 1 1
NOAA10 1 2
NOAA7 1 2
NOAA7 1 3
NOAA11 4
G. caribaeus
0.08 substitutions per site

Figure 10

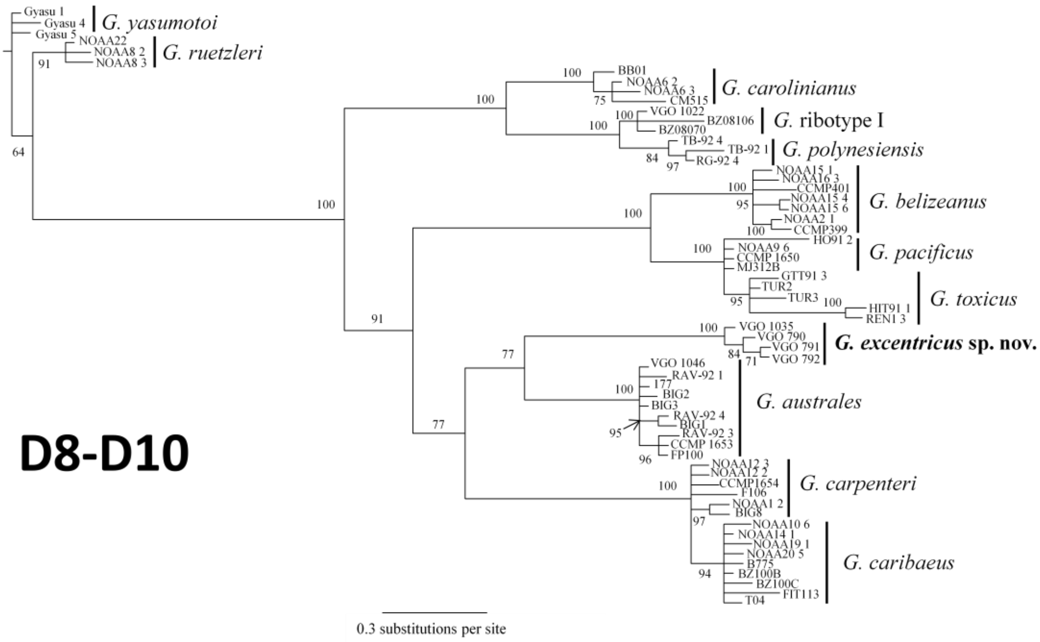
Gyasu 1
Gyasu 4
Gyasu 5
G. yasumotoi
NOAA22
NOAA8 2
NOAA8 3
G. ruetzleri
91
64
100
100
BB01
NOAA6 2
NOAA6 3
CM515
75
G. carolinianus
100
VGO 1022
BZ08106
BZ08070
100
G. ribotype I
TB-92 4
TB-92 1
RG-92 4
84
97
G. polynesiensis
100
NOAA15 1
NOAA16 3
CCMP401
NOAA15 4
NOAA15 6
NOAA2 1
CCMP399
100
95
100
G. belizeanus
100
HO91 2
NOAA9 6
CCMP 1650
MJ312B
100
G. pacificus
GTT91 3
TUR2
TUR3
HIT91 1
REN1 3
95
100
G. toxicus
91
VGO 1035
VGO 790
VGO 791
VGO 792
100
84
71
G. excentricus sp. nov.
77
VGO 1046
RAV-92 1
177
BIG2
BIG3
RAV-92 4
BIG1
RAV-92 3
CCMP 1653
FP100
100
95
96
G. australes
77
NOAA12 3
NOAA12 2
CCMP1654
F106
NOAA1 2
BIG8
100
97
G. carpenteri
NOAA10 6
NOAA14 1
NOAA19 1
NOAA20 5
B775
BZ100B
BZ100C
FIT113
T04
94
G. caribaeus
D8-D10
0.3 substitutions per site

Figure11

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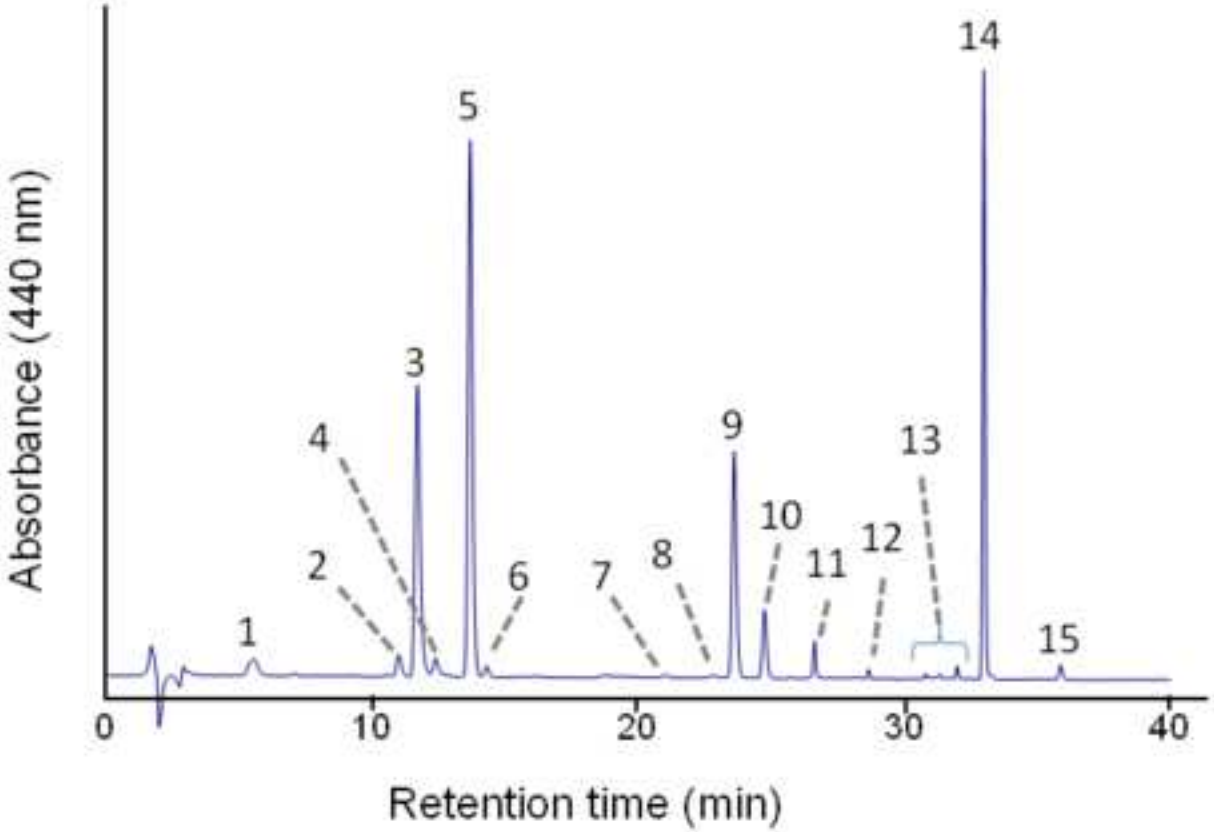

Table 1
CTX- and MTX-like toxicity estimated using the Neuroblastoma cell-based assay.

CTX-like toxicity

Strain	IC$_{50}$ $^{O/V-}$ ±SD (cells eq. mL^{-1})	IC$_{50}$ $^{O/V+}$ ±SD (cells eq. mL^{-1})	p value (*t* test)	pg CTX 1B eq cell^{-1} ±SD
VGO790	2.11 ± 0.16	0.87 ± 0.10	0.001	1.10 ± 0.19
VGO791	1.60 ± 0.28	0.65 ± 0.23	0.01	1.05 ± 0.18
VGO792	4.58 ± 0.86	2.35 ± 0.77	0.001	0.37 ± 0.17

MTX-like toxicity

Strain	IC$_{50}$ $^{SK\&F\ 96365-}$ ±SD (cells eq. mL^{-1})	IC$_{50}$ $^{SK\&F\ 96365+}$ ±SD (cells eq. mL^{-1})	p value (*t* test)	Dose-ratio (DR)	ng MTX eq cell^{-1} ±SD
VGO790	7.73 ± 0.64	28.81 ± 5.97	0.001	3.73	1.38 ± 0.31
VGO791	14.4 ± 0.33	68.99 ± 24.88	0.02	4.79	0.60 ± 0.24
VGO792	19.78 ± 3.62	71.51 ± 19.43	0.01	3.62	0.48 ± 0.16