Distribution, identification and cytotoxicity of *Gambierdiscus* Dinophyceae) in the Atlantic Selvagens Islands (Madeira, Portugal): a ciguatera gateway to Europe

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ABSTRACT

The emerging threat of ciguatera poisoning (CP) in Europe has been associated with fish captured in the Canary Islands (Spain) and Selvagens Islands (Portugal). The first are heavily populated islands where numerous scientific studies have been carried out. Conversely, the Selvagens Islands are a nature reserve with low human pressure that have been rarely surveyed in terms of the marine benthic microalgae, including the epiphytic ciguatera-causing dinoflagellate species. To investigate the harmful microalgal diversity of the Selvagens Islands, a scientific cruise to these remote islands took place in September, 2018. The *Gambierdiscus* species composition and distribution, and the associated epiphytic dinoflagellate community, were assessed using artificial substrate devices. *Gambierdiscus* cells were found in all samples, reaching concentrations of up to 725 cells 100 cm–2. *G. australes* was the only species identified after morphological and molecular analysis of the retrieved cultures. Species identification was confirmed by molecular characterization based on the LSU D8– D10 region. Nevertheless, phylogenetic studies indicated that some strains diverged from the *G. australes* clade suggesting genetic differentiation. Toxicity was estimated by neuro-2a cell-based assay in four strains, ranging from 2.46–83 fg of CTX1B eq. cell–1. The epiphytic dinoflagellate community that co occurred with *Gambierdiscus* comprised other toxic or potentially toxic dinoflagellates, such as *Ostreopsis, Prorocentrum, Amphidinium* and *Coolia* species. Oceanographic and meteorological data were also obtained to characterize the occurrence of *Gambierdiscus*. This study is the first stage in understanding the role of the Selvagens Islands in the incubation and proliferation of the ciguatera-causing dinoflagellates *Gambierdiscus* in the NE Atlantic.

HIGHLIGHTS

- The Selvagens Islands are a ciguatera hotspot in Europe.
- Gambierdiscus australes was the only species observed in the Selvagens Islands.
- Strains diverging from the G. australes clade suggest genetic differentiation.

Introduction

The Selvagens Islands are a unique ecosystem in the NE Atlantic with an outstanding ecological and natural value of well-preserved marine and terrestrial biota. These nearly inaccessible small volcanic islands are located between the Madeira and Canary archipelagos, being the core of the Macaronesia region. The islands were classified as a nature reserve in 1971 to protect the world's largest breeding colony of Cory's shearwater *Calonectris borealis*, and other threatened

seabird species, in a total marine area of 95 km2, which is delimited by the 200 m depth bathymetry line. Extension of the current limit of 200 m depth, that can be reached in a few hundred metres from the coastline due to the narrow and steep insular shelf, was recommended by the National Geographic Society (NGS) after a scientific expedition carried out in May 2015 (Friedlander *et al*., 2016). These islands were considered one of the last remaining intact marine ecosystems in the eastern Atlantic and designated by the NGS as one of the pristine sites of the oceans today (Friedlander *et al*., 2016). Commercial and recreational fishing are prohibited in the Selvagens Islands marine protected area, but some exceptions, such as spearfishing and angling, were authorized in the reserve until 2008. By this time a total ban on fisheries was issued due to the risk of ciguatera poisoning (CP). CP is a food-borne illness caused by the consumption of fish and fisheries products containing the potent neurotoxins named ciguatoxins (CTX). CTX act at the voltagegated sodium channels (VGSC), increasing the ion permeability and cell disruption which leads to persistent neurological impairment (Bidard *et al*., 1984; Lombet *et al*., 1987; Nicholson & Lewis, 2006). These toxins are traditionally associated with coral reef fish species from tropical and subtropical regions, with CP being one of the most common types of food poisoning associated with the consumption of fish. Cases of CP in European populations were historically limited to travellers to endemic areas or due to the consumption of imported fish. Nowadays CP is an emerging threat in Europe. The first outbreak occurred relatively recently in the Canary Islands, Spain, in 2004 (Pérez-Arellano *et al*., 2005). Since then several human intoxications have been reported in Macaronesia (Soliño & Costa, 2020). In many cases, the poisonings were caused by fish caught in the surrounding waters of the Selvagens Islands (Boada *et al*., 2010; Otero *et al*., 2010; Estevez *et al*., 2019), where relatively high CTX levels have been determined in fish from different positions in the trophic chain (Costa *et al.*, 2018, 2021). CTXs are produced by the epiphytic benthic dinoflagellates *Gambierdiscus* and *Fukuyoa* (Bagnis *et al*., 1980; Yasumoto, 2001) but can also be products of fish metabolism resulting from biotransformation of precursor compounds produced by these dinoflagellates (Lewis & Holmes, 1993; Ikehara *et al*., 2017). *Gambierdiscus* species were first described from the Gambier Islands, French Polynesia (Yasumoto *et al*., 1977), where the high incidence of ciguatera affects thousands of people every year (Skinner *et al*., 2011). In the Atlantic Ocean, *Gambierdiscus* spp. are widespread in the Caribbean Sea and associated with high rates of CP (Boucaud-Maitre *et al*., 2018). In the eastern Atlantic Ocean, these dinoflagellates were described for the first time in Cape Verde Islands as *Goniodoma* sp. by E.S.Silva in 1948 (Silva, 1956; Fraga *et al*., 2011). *Gambierdiscus* and *Fukuyoa* grow preferably in shallow waters with temperatures higher than 21°C, high and stable salinities, and relatively low light intensities (Parsons *et al*., 2012). Moreover, the epiphytic dinoflagellate assemblages that co-occur with *Gambierdiscus* species were associated in the past with ciguatera fish poisoning, possibly playing a role in *Gambierdiscus* distribution, such as developing strategies against grazing by producing toxins or mucilage to form large aggregates (Skinner *et al*., 2013). The increasing occurrence and spread of *Gambierdiscus* to temperate regions seems to be favoured by climate warming trends (Kibler *et al*., 2015). As a result of climate change or due to increased monitoring efforts coincident with CP outbreaks, *Gambierdiscus* spp. Have been detected in subtropical-temperate regions, such as the Canary Islands, Madeira and the Mediterranean Sea (Aligizaki & Nikolaidis, 2008; Fraga *et al*., 2011; Reverté *et al*., 2018; Hoppenrath *et al*., 2019; Tudó *et al*., 2020*a, b*). A large number of *Gambierdiscus* species have been recorded in the Canary Islands, where studies revealed the presence of *G. australes, G. caribaeus, G. carolinianus, G. excentricus, G. belizeanus* and *G. silvae*, with notably higher abundance in the eastern islands of the archipelago (Fraga & Rodríguez, 2014; Rodríguez *et al*., 2017; Bravo *et al*., 2019; Tudó *et al*., 2020*a*). In contrast, single species composition was observed after opportunistic sampling in the Selvagens and Madeira Islands, where *G. australes* and *G. excentricus* were detected, respectively (Reverté *et al*., 2018; Hoppenrath *et al*., 2019). Responding to the urgent need to investigate the risk of ciguatera in Europe, a scientific cruise was carried out in September 2018 to the Selvagens Islands where relevant levels of CTX have

been detected in fish (Costa *et al.*, 2018, 2021). This study reports the results of the investigation carried out to assess the *Gambierdiscus* distribution, species composition, and the associated epiphytic dinoflagellate community in this rarely surveyed marine protected area of the NE Atlantic.

Materials and methods

Study area

The Selvagens Islands are located in the temperatesubtropical North-east Atlantic, at 293 km south-east of Madeira Island (Portugal), 180 km north of Tenerife Island (Spain) and 600 km west of the African continental coast of Morocco (Fig. 1). They are the oldest archipelago (29.5 Ma) of the Macaronesian islands, composed by the main island Selvagem Grande, a table-top island of area 2.4 km$_2$, and a group of small islands, with the largest being Selvagem Pequena (0.2 km$_2$), islets and reefs (Geldmacher *et al.*, 2001). The islands are shaped by marine abrasión (Supplementary figs S1, S2), with occasional strong east winds from the African continente transporting large quantities of sand particles and high temperatures. The islands' small size and low altitude (163 m highest altitude) are not propitious for condensation and annual precipitation levels are low. In both islands two sampling stations were selected, Cagarras Bay and Galinhas Bay in Selvagem Grande and Espanhóis Bay and Fundeadouro in Selvagem Pequena. The samples were collected during a cruise in September 2018 (Fig. 1).

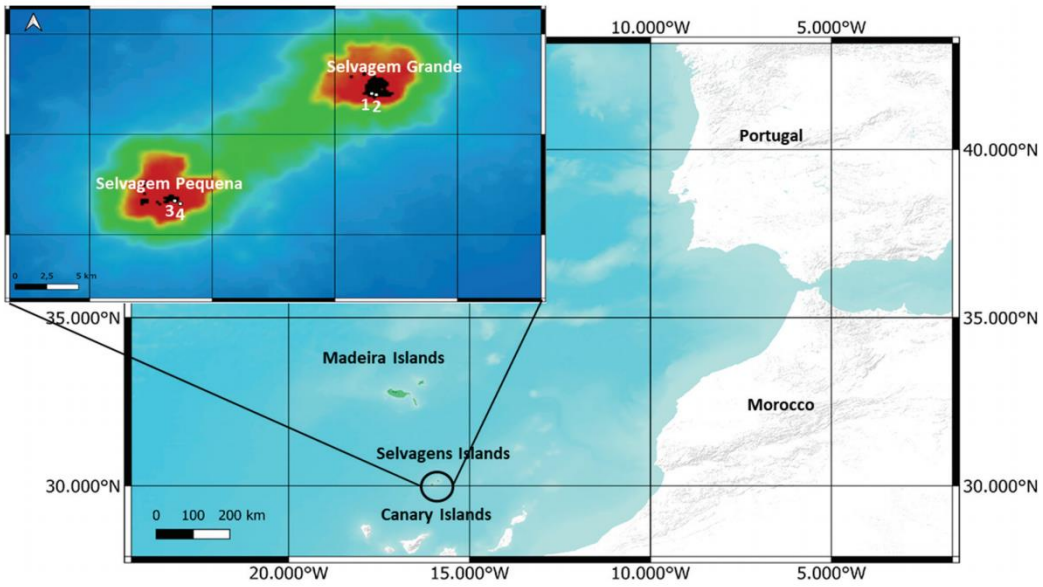

Fig. 1. Location of sampling sites in the Selvagens Islands (Madeira, Portugal): 1 – Cagarras Bay (30°08′26.6″N, 15° 52′11.5″W), 2 – Galinhas Bay (30°08′22.2″N, 15°51′58.3″W), 3 – Espanhóis Bay (30°02′01.3″N, 16°01′50.0″W), 4 – Fundeadouro (30°01′51.1″N, 16°01′33.5″W).

Table 1. *Gambierdiscus* strains isolated from artificial substrate samples, sampling conditions and GenBank accession numbers of the strains used for the phylogenetic analysis.

Strain code	Species	Sampling date	Sampling site	Incubation time (hours)	Depth (m)	GenBank accession number
IPMA_GAMBI6_SG_18	*G. australes*	15-06-2018	Selvagem Grande Cagarras Bay	48	7	OM141582
IPMA_GAMBI16_SG_18	*G. australes*	05-09-2018	Selvagem Grande Cagarras Bay	48	7	OM141578
IPMA_GAMBI17_SG_18	*G. australes*	05-09-2018	Selvagem Grande Cagarras Bay	48	7	OM141589
IPMA_GAMBI18_SG_18	*G. australes*	05-09-2018	Selvagem Grande Cagarras Bay	48	7	OM141588
IPMA_GAMBI19_SG_18	*G. australes*	05-09-2018	Selvagem Grande Cagarras Bay	48	7	OM141581
IPMA_GAMBI20_SG_18	*G. australes*	07-09-2018	Selvagem Grande Cagarras Bay	48	3	OM141579
IPMA_GAMBI2_SG_18	*G. australes*	07-09-2018	Selvagem Grande Cagarras Bay	48	3	OM141583
IPMA_GAMBI4_SG_18	*G. australes*	15-06-2018	Selvagem Grande Cagarras Bay	48	7	OM141584
IPMA_GAMBI23_SG_18	*G. australes*	07-09-2018	Selvagem Grande Cagarras Bay	48	3	OM141577
IPMA_GAMBI33_SG_18	*G. australes*	07-09-2018	Selvagem Grande Cagarras Bay	48	3	OM141585
IPMA_GAMBI4_SP_18	*G. australes*	06-09-2018	Selvagem Pequena Espanhóis Bay	24	7	OM141586
IPMA_GAMBI13_SP_18	*G. australes*	07-09-2018	Selvagem Pequena Espanhóis Bay	24	7	OM141587
IPMA_GAMBI6_SP_18	*G australes*	07-09-2018	Selvagem Pequena Espanhóis Bay	24	7	OM141590
IPMA_GAMBI4_BG_18	*G. australes*	07-09-2018	Selvagem Grande Galinhas Bay	48	7	OM141580

Benthic dinoflagellate community sampling

In order to sample benthic and epiphytic dinoflagellates an artificial substrate was used based on currently accepted sampling strategies (Tester *et al*., 2014; Parsons *et al*., 2017). The artificial substrate was made of rectangular pieces of mosquito net (1.6 × 1.8 mm porosity). The substrates (n = 3) were deployed in each sampling point, at 1 m above the bottom and 3 or 7 m below the sea surface attached to a weight and a small subsurface buoy, ensuring that they were held tightly on both sides and naturally positioned perpendicularly to the water flow. Incubation times followed those used by Tester *et al*. (2014) as closely as possible but due to trip logistics, incubation times differed in Selvagem Grande and Selvagem Pequena. Mosquito nets were incubated for 24 h in Selvagem Pequena, and 48 h in Selvagem Grande, after which they were collected with the surrounding seawater using plastic bags and shaken vigorously for 10 s to detach the epiphytic and benthic microalgae cells from the net (Jauzein *et al*., 2016). Then, water was passed through a 500 µm Meshed filter. The total volume obtained for each sample was recorded. An aliquot of seawater taken from all samples was used for in site isolation of *Gambierdiscus*, using a Zeiss Axiovert 40C (Göttingen, Germany), and then all samples were preserved with Lugol iodine solution (1% final concentration) for subsequent quantification of epiphytic dinoflagellate cells. The samples were stored in brown plastic bottles until analysis and processed in les than a month. The enumeration of *Gambierdiscus* cells and the epiphytic dinoflagellates that co-occur in all samples was accorded by the Utermöhl method (Utermöhl, 1958) using an inverted microscope Leica DMi8 manual (Leica Microsystems CMS GmbH, Wetzlar, Germany) at 200× magnification with detection limit of 20 cells l^{-1}. Cell abundance in the artificial substrate were expressed as cells per 100 cm^2 according to Tester *et al*. (2014).

Gambierdiscus *cultivation and identification*

Isolated cells were transferred and maintained in a 4-well microplate with sterile-filtered seawater enriched with f/2-medium (Guillard & Ryther, 1962) at 23°C ± 1 under 50 µmol photon m^{-2} s^{-1} and 12:12 light:dark (Table 1). After six weeks of slowly growing, a monoclonal culture

was established and living cells or empty theca of interest were picked and placed on an object slide and observed using a Leica DMi8 automated inverted microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) equipped with differential interference contrast optics and fluorescence at 400× magnification. Digital photos were taken using a Leica DFC7000 T camera (Leica Microsystems CMS GmbH, Wetzlar, Germany). For scanning electron microscopy (SEM), living cells were fixed with Lugol iodine solution and placed on a 4 µm PC membrane Nuclepore filter (Millipore, Bedford, Massachusetts, USA) rinsed with tap water several times and air dried at room temperature (22°C). The filter was mounted on a stub and sputter coated with gold-palladium and observed using a JEOL JSM-5200 scanning microscope. Labelling of thecal plates follows the traditional Kofoid system of plate series (Kofoid, 1909; Chinain *et al*., 1999).

DNA extraction, amplification and sequencing

One sample of 2 ml from each *Gambierdiscus* culture (Table 1) was harvested and centrifuged at 15 000 rcf. The DNA was released from the pellets by adding the dilution buffer of Phire Plant Direct PCR Kit (Thermo Scientific). A DNA fragment of 900 bp within the LSU D8-D10 region was amplified with the primers FD8 (5' – GGATTGGCTCTGAGGGT TGGG – 3') and RB (5'– GATAGGAAGAGCCGAC ATCGA – 3') (Chinain *et al*., 1999) at a final concentration of 0.5 µM. The amplifications were performed in a reaction mixture of Phire master mix with Hot Start II DNA Polymerase (Thermo Scientific) using a T100™ Thermal Cycler (BioRad) programmed with a PCR cycle consisting of an initial denaturation step at 98°C for 5 min, followed by 35 cycles of 5 s at 98°C, 5 s at 64.1°C and 20s at 72°C and a final extension step of 1 min at 72°C. The amplified fragments were visualized under UV light after electrophoretic analysis performed in 1.5% w/v agarose gel with GreenSafe Premium™ DNA staining (NZYTech), at 75 V in 0.5× Tris-borate EDTA (TBE) buffer for 40 min. The PCR products were purified and sequenced by Sanger sequencing in the commercial platform GATC Biotech (Eurofins Genomics).

Phylogenetic analyses

The phylogenetic analyses were performed using the curated dataset available in Kretzschmar *et al*. (2019) and aligned with the sequences from our study using MUSCLE v3.8.31 (Edgar, 2004) and trimmed using Gblocks v0.91b (Castresana, 2000) resulting in 518 selected positions. Maximum likelihood phylogeny was calculated with PhyML v3.1 (Guindon & Gascuel, 2003) using a HKY85 substitution model and tree support by calculating 1000 bootstraps using Gamma distribution. Bayesian inference to estimate the posterior probability distribution was calculated using MrBayes v3.2.7a (Huelsenbeck & Ronquist, 2001). The number of substitution types was fixed to 6. Four Markov Chain Monte Carlo (MCMC) chains were run for 150 000 generations, sampling every 100 generations, with the first 1000 sampled trees discarded. Genetic distances were calculated within the genus and between species with the LSU rDNA D8–D10 region using *p* distance uncorrected genetic distance (UGD) with pairwise deletion in MEGA X (Nishimura *et al*., 2013; Kumar *et al*., 2018). GenBank accession numbers of the DNA sequences obtained in this study are presented in Table 1.

CTX-like toxicity measured by neuroblastoma (Neuro-2a) cell-based assay (CBA)
Gambierdiscus *cultures for toxicity determination*

The strains GAMBI4_SP_18 and GAMBI6_SP_18 from Selvagem Pequena, and GAMBI2_SG_18 and GAMBI4_BG_18 from Selvagem Grande, were scaled-up to non-treated 260 and 645 ml Nunc sterile culture flasks for biomass production (Fisher Scientific, Porto Salvo, Portugal). The culture was performed under the same conditions previously described. Flasks were routinely counted for culture stage surveillance and cells were harvested at the early stationary phase by filtration under vacuum onto GF/F glass microfibre filters (Whatman, Lisbon, Portugal) and extracted as described elsewhere (Pisapia *et al*., 2017). Briefly, filters were covered with absolute MeOH (~10 ml per million of microalgae), sonicated for 15 min at 25 W, 50% pulse duty cycle (Vibra-cell, Sonic & Materials, Newtown, Connecticut, USA) while cooling in an ice bath and subsequently centrifuged (4000 rpm, 10 min). These steps were repeated twice with 50% MeOH and supernatants were combined. During the extraction procedures, disruption of cells was checked under an inverted microscope Zeiss IM35 (Filsat, Porto, Portugal). The final extract was evaporated at a maximum 60°C under vacuum and sent to IRTA for toxicity assessment.

Cell based assay

For cell maintenance, Neuro-2a cells (ATCC, CCL131) were cultured in 10% foetal bovine serum (FBS) RPMI medium with 1% sodium pyruvate solution (100 mM), 1% l-glutamine solution (200 mM), and 0.5% antibiotic solution (10 mg ml^{-1} streptomycin and 1000 U ml^{-1} penicillin) (Sigma–Aldrich, USA). Cultures were maintained at 37°C and 5% CO_2 in a humid atmosphere incubator (Binder, Tuttlingen, Germany). The day prior to the assay, cells were seeded in a 96-well microplate in 200 µl of 5% FBS-RPMI medium at a density of 35 000 cells per well. Cells were incubated under the same conditions as described for cell maintenance. Every standard (CTX-1B) and sample extract was assayed in triplicate. Half the wells of each microplate were pre-treated with ouabain and veratridine corresponding to a final concentration of 1 and 0.1 mM, respectively. Standard solutions and sample extracts were reconstituted in RPMI 5%. Then, samples were serially diluted, and 10 µl added to each well, with (O/V+) and without (O/V-) ouabain and veratridine (Merck, Germany) pre-treatment. On the next day, cell viability was measured, by means of the MTT test [3-(4,5-dimethylthiadol-2-yl)-2,5-diphenyltetrazoliu-m] (500 µg ml^{-1}) (Manger *et al*., 1993) and absorbance measured at 570 nm using automated multi-well scanning. Samples were considered as positive when cell viability was inhibited in O/V+ wells and unaffected in O/V-wells. The LOQ calculated for each trial varied from 0.0021 and 0.0077 pg $cell^{-1}$. The purified CTX1B (2 µg ml^{-1}) used for the CBA calibration curves was obtained from Prof. Richard J. Lewis (The Queensland University, Australia) and cross-calibrated in relation to the NMR-quantified CTX1B reference material provided by Prof. Takeshi Yasumoto (Japan Food Research Laboratories, JFRL).

Results

Distribution and quantification of Gambierdiscus and its co-occurring epiphytic dinoflagellate community of the Selvagens Islands

Gambierdiscus cells attached well to mosquito nets at both sites. *Gambierdiscus* was present in all sampling points in both islands, although in higher concentrations in Selvagem Grande. *Gambierdiscus* sp. abundance in artificial substrates ranged between 294–1302 cells 100 cm^{-2} in Selvagem Grande, and between 223–725 cells 100 cm^{-2} in Selvagem Pequena. The increased period of incubation in Selvagem Grande (48 h) led to strong colonization of the artificial

substrate by benthic diatoms, including *Thalassiosira* sp., *Licmophora* sp., *Navicula* sp. and *Diploneis* sp. These diatoms almost completely filled the artificial substrate in Selvagem Grande. It was still possible to quantify *Gambierdiscus* cells but made it imposible to harvest and accurately count other benthic dinoflagellates. For this reason only dinoflagellates with cell lengths greater than 60 µm were counted. In Selvagem Pequena it was possible to assess the associated epiphytic dinoflagellate community where *Ostreopsis* sp. was dominant representing 54.1%. The other observed genera were *Prorocentrum* spp., and *Amphidinium* spp., representing 26.3% and 5.7% respectively of the epiphytic dinoflagellate community. Within the *Prorocentrum* and *Amphidinium* genera it was possible to identify some organisms at the species level by light microscopy, namely *P. hoffmanianum, P. panamense, P. lima, P. concavum, P. emarginatum, P. rhathymum* and *A*. *carterae* (Supplementary fig. S3).

Gambierdiscus *morphological observations*

Based on light and scanning microscopy *Gambierdiscus* cells in culture were round to ellipsoid shape in apical view and compressed anteroposteriorly with brown chloroplasts (Fig. 2). The thecal plates follow the Po, 3', 7", 6c, 8s, 5"', 1p, 2"" formula. Variability of cell size is presented in Table 2 and illustrated in Fig. 3. Smooth cell surface and convex valves were observed. The thecal pores were numerous and round with a smooth edge. The apical pore plate (Po) was a broadly ellipsoid plate with a fish-hook shaped opening surrounded by 31 pores (mean pore diameter of 0.38 µm). The 2' apical plate size ranged from 33.11– 1.58 µm, being the largest plate of the epithecal plates. The hypotheca has 8 hypothecal plates, 1"" and 2"" plates were small and quadrangular, lying parallel to the 1p plate at the dorsoventral axis of the cell. The 1p plate was long and narrow. Plates 1"' and 5"' were the smallest, positioned close to the cingulum. Based on these morphological discriminating characters the species were identified in samples from both islands as *Gambierdiscus australes*, as described in Chinain *et al*. (1999).

Molecular characterization and phylogeny

The phylogeny of *Gambierdiscus* species based on the LSU D8-D10 region is presented in Fig. 4. Tree topology was similar for both ML and BI analysis and congruent with previous studies (Litaker *et al*., 2010; Nishimura *et al*., 2013; Kretzschmar *et al*., 2019). The strains isolated from the Selvagens Isla-nds clustered with the *G. australes* clade, with *Gambierdiscus* sp. (VGO1258; Rodríguez *et al*., 2017) from the Canary Islands as its sister clade and *G. excentricus* the closest relative in this subtree. Most of the strains were identical to *G. australes* from the Australes archipelago type material (*G. australes* RAV2; Chinain *et al*., 1999; Litaker *et al*., 2009). However, the strains GAMBI13_SG_18, GAMBI18_SG_18 and GAMBI17_SG_18 from Selvagem Grande diverge from the other *G. australes* with a bootstrap support for ML analysis of 74. The BI posterior probability support value for this node was below 0.70. The strains GAMBI13_SG_18 and GAMBI18_SG_18 diverged with stronger support values of 75 for ML and 0.93 for BI posterior probability (Fig. 4). A more detailed analysis of the *G. australes* clade was performed using 57 sequences from several geographic locations available in the GenBank database (Fig. 5). Here, the clade consisting of the strains GAMBI17_SG_18 and GAMBI18_SG_18 maintained its divergence from the other *G. australes* with higher support (83ML/0.83BI). The strain GAMBI13_SG_18 showed no divergence from the other *G. australes*. Furthermore, the higher genetic distance (*p*-distance) obtained within *G. australes* strains was for GAMBI17_SG_18 with a mean distance of 0.0377± 0.00741 and an inter-species distance of 0.0513 from *Gambierdiscus* sp. VGO1258 and 0.0882 from *G. excentricus* (Supplementary table S1). The mean genetic distance for GAMBI18_SG_18 was 0.0255 ±0.00858 and inter-species distance of 0.0376 from *Gambierdiscus* sp. VGO1258 and 0.0725 from *G. excentricus*.

CTX-like toxicity

Four strains of *Gambierdiscus australes* isolated from both Selvagem Grande and Selvagem Pequena were cultivated for toxicity determination by Neuro-2ª cell-based assay. Low to moderate toxicity was found for these strains (Table 3, Supplementary fig. S4). CTX-like toxicity ranged from 2.46–83 fg CTX1B eq. $cell_{-1}$, being the most toxic strain, the strain GAMBI4_SP_18 isolated from Selvagem Pequena.

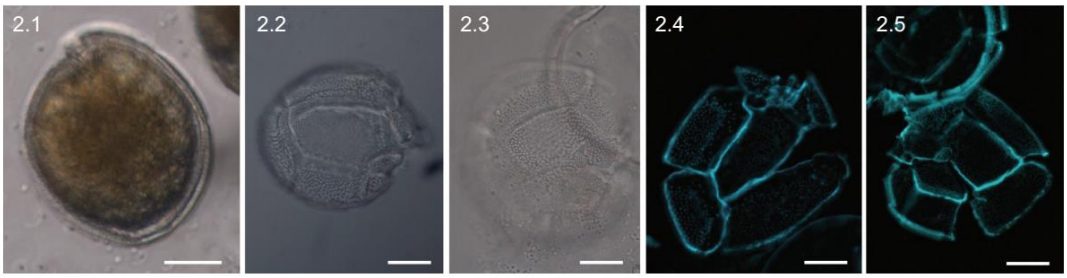

Fig. 2. Photomicrographs representing *Gambierdiscus australes* cultured from Selvagens Islands. 2.1: *Gambierdiscus* living cells from cultures; 2.2 and 2.3: bleached on observation cells of *G. australes* in light microscopy and 2.4 and 2.5: calcofluor staining of bleached theca in fluorescence microscopy at 640×. Scale bar 25 µm.

Table 2. Cell size estimated as length (L), width (W), and ratio of length and width (L/W), dimensions of apical pore plate (Po) and surrounding pore numbers and diameter, size of 1p and 2' plate. Arithmetic mean and standard deviation (SD) of *Gambierdiscus australes* from Selvagem Grande and Selvagem Pequena.

	Cell size (n = 38)			Apical plate poro (Po) (n = 38)			2' plate size (n = 38)			Pore (n = 38)		1' plate size (n = 23)		
	L	W	L/W	L	W	L/W	L	W	L/W	Number	Ø	L	W	L/W
Mean	77.0	73.9	1.0	6.9	4.8	1.5	43.2	21.9	2.0	30.7	0.4	51.7	30.3	1.7
SD	5.9	5.27	0.1	0.7	0.7	0.2	5.4	3.5	0.4	1.1	0.1	5.7	5.1	0.3

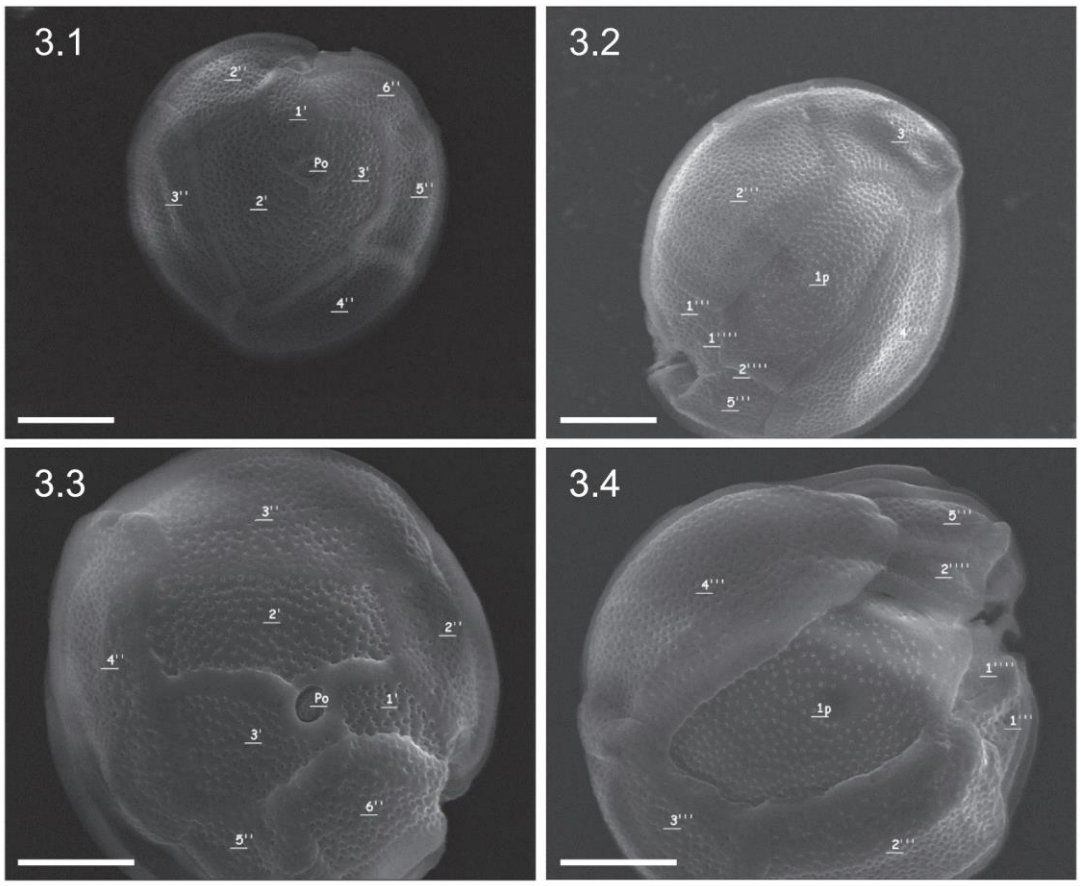

Fig. 3. Scanning electron micrographs of *Gambierdiscus australes* in Selvagens islands. 3.1, 3.2: apical and antapical view of the epitheca and hypotheca respectively showing the tabulation of *Gambierdiscus* from Selvagem Pequena island at 1000× magnification; 3.3, 3.4: apical and antapical view of the epitheca and hypotheca respectively showing the tabulation of *Gambierdiscus* from Selvagem Grande Island at 1500× magnification. Scale bar 20 µm.

Discussion

This is the first study quantifying the abundance of ciguatera-causing dinoflagellates in Selvagens Islands. *Gambierdiscus* were detected in substrates deployed in both islands. The abundance on artificial substrates reached equilibrium with the surrounding population within 24 h, even though slightly higher *Gambierdiscus* densities can be attached to the substrates within 48 h (Tester *et al*., 2014). *Gambierdiscus* cell densities up to 1302 cells 100 cm–2 were observed, which are higher tan those found by Tester *et al*. (2014) in Belize and Malaysia, and similar or lower than that found by Fernández-Zabala *et al*. (2019) in the nearby archipelago of the Canary Islands. *Gambierdiscus australes* was the only species clearly identified from the substrate, which is in agreement with previous findings (Reverté *et al*., 2018). The presence of *G. australes* in the Selvagens Islands confirms its dispersionalong the Macaronesian region. Morphometric values of *G. australes* from the Selvagens Islands matched well with those of *G. australes* in the

Canary and Balearic Islands (Bravo *et al*., 2019; Tudó *et al*., 2020*b*), as well as *G. australes* from French Polynesia and the Pakistan waters (Chinain *et al*., 1999; Litaker *et al*., 2009; Munir *et al*., 2011). The molecular analysis also supported the species identity. According to the classic 'Island Biogeography Theory' proposed by MacArthur & Wilson (1963) and revisited by Warren *et al*. (2015), species richness can be related to the size of the islands and their distance to the mainland. Higher *Gambierdiscus* cell concentrations have been observed in the easternmost Canary Islands (i.e. Lanzarote and Fuerteventura) located closer to the Morocco coast tan westernmost islands where a reduced number of *Gambierdiscus* species and lower cell concentrations have been reported (Rodríguez *et al*., 2017). Only one *Gambierdicus* species was found, until now, in the small sized and isolated Selvagens Islands. A few strains from both Selvagem Grande and Selvagem Pequena were found to diverge from the *G. australes* clade indicating genetic differentiation of these strains. The genetic distances are within the range for inter-species differentiation reported for other studies (Litaker *et al.*, 2009, 2010; Nishimura *et al.*, 2013, 2014; Smith *et al*., 2016) and higher than intra-species distance obtained by Nishimura *et al*. (2013) (range: 0.001–0.008). Nevertheless, the genetic distances obtained within the *G. australes* strains excluding GAMBI17 and GAMBI18_SG_18 were on average 0.0074±0.00672, reaching the upper limit reported by Nishimura *et al*. (2013), and the *G. australes* species tree showed that several clades formed within the species and two of them with high support values indicating intra-species divergence. The observed divergences within the LSU may be the result of artefacts caused by pseudogenes as reported by other authors (Litaker *et al*., 2009). In addition, the Fig. 5. Phylogenetic análisis of *Gambierdiscus australes* based on D8-D10 LSU rDNA region. Node support values are bootstrap/Bayesian posterior probability obtained from Maximum likelihood and Bayesian inference analysis respectively, only the values with support above 80 are presented. The species sequenced from this study are in bold type. GenBank accession numbers from these sequences are displayed in Table 1. The sequences used to construct the phylogeny are presented with the species name, strain code and GenBank database identifier. GenBank identifiers of redundant sequences are presented in Supplementary table S2. Scale bar is substitutions per site. rRNA genes evolve more slowly tan protein coding genes and the variable domains tend to diverge during speciation (Litaker *et al*., 2009). Although this study does not provide specific evidence to support species differentiation, the geographic isolation and benthic lifestyle may prevent gene flow and favor the continuous speciation process until genetic differences stabilize and a different species can be recognized (Presgraves & Glor, 2010). The effects described in the 'Island Biogeography Theory' may be expected in these islands but *in situ* speciation must also be taken into consideration. According to Presgraves & Glor (2010), 'Taxa able to accumulate population genetic differentiation on small geographic scales should also show small minimum threshold áreas and higher probabilities of speciation'. Our study, alongside others, reveals Macaronesia as an important site to understand the full genetic diversity of *Gambierdiscus* sp. (Fraga & Rodríguez, 2014; Rodríguez *et al*., 2017; Bravo *et al*., 2019; Hoppenrath *et al*., 2019; Tudó *et al*., 2020*a*). Rodríguez *et al*. (2017) described a strain from Tenerife (Canary Islands) genetically different from the rest of the *G. australes* clade, which almost certainly represents a new species. A comprehensive understanding of the toxic benthic marine microbial eukaryote will require further studies and assessment of results from a combination of molecular techniques and different markers, as described by Smith *et al*. (2017), Verma *et al*. (2020) and Chomérat *et al*. (2020). CTX-like toxicity was previously investigated in *Gambierdiscus* strains from the Selvagens and Canary Islands, including six *G. australes* strains from the Selvagem Grande (Reverté *et al*., 2018). In their study, toxicity is reported for five of the six strains analysed and varies between 200 and 515 fg CTX1B eq. $cell^{-1}$, which is considerably higher tan the toxicity found in the present study. The CTX levels

observed in GAMBI4_SP_18 are in the range of *G. excentricus* strains (VGO790, VGO791) and *G. australes* strains (IRTA-SMM-13-09, IRTA-SMM -13-10) isolated in Canary Islands (Caillaud *et al*., 2010; Fraga *et al*., 2011; Reverté *et al*., 2018). The lower CTX potency of strains GAMBI6_SP_18, GAMBI2_SG_18 and GAMBI4_BG_18, closely related with GAMBI4_SP_18 reflects the high variability in toxicity in this species, which has also been found in previous studies (Chinain *et al*., 2010; Reverté *et al*., 2018; Rossignoli *et al*., 2020). Despite differences in Neuro-2a cytotoxicity, the present study confirms the CTX-like toxicity of *G. australes* from the Selvagens Islands. If this species is confirmed to be the primary source of CTXs in these islands, then it should be investigated which conditions promote higher toxicity and the full suite of toxins characterized to better understand the flux of CTX in the environment. Several other benthic and epiphytic dinoflagellates were observed co-existing with *Gambierdiscus* in Selvagens Islands, including potentially toxic species of the genera *Ostreopsis, Prorocentrum, Amphidinium* and *Coolia* (Supplementary fig. S2). Benthic communities with such general assemblage of dinoflagellates have been reported in the nearby Canary Islands and several other geographic locations, such as Hawaii and islands of the Mesoamerican Reef System of the Mexican Caribbean (Parsons & Preskitt, 2007; Fraga & Rodríguez, 2014; Irola-Sansores *et al*., 2018; Bravo *et al*., 2020). The proliferation of *Gambierdiscus* as well as of the remaining species enumerated here depends on poorly understood ecological processes, eventual species interactions, and a complex combination of factors that must be investigated. The Selvagens Islands are a stable environment with smooth seasonal variations where seawater temperature does not typically decrease below 18°C at the peak of winter months and reaches the optimal temperature for *Gambierdiscus* growth (>24°C) between August–October (Supplementary figs S1–S2). Co-existence may require niche differences tominimize competition but this study and previous findings (Fraga & Rodríguez, 2014; Bravo *et al*., 2020) suggest the coexistence of congeneric species with the same ecological niche. The Selvagens Islands, characterized by high abundances and biodiversity of marine life, may play a role in incubation and proliferation of CP-causing dinoflagellates and other toxic benthic microalgae. The occurrence of CP in Europe is highlighting the Selvagens Islands as an archipelago with the required features for development of epiphytic benthic harmful algal blooms. Moreover, these islands present interesting characteristics to investigate *in situ* the evolution of ecosystems functioning where Selvagem Grande and Selvagem Pequena may act as replicates.

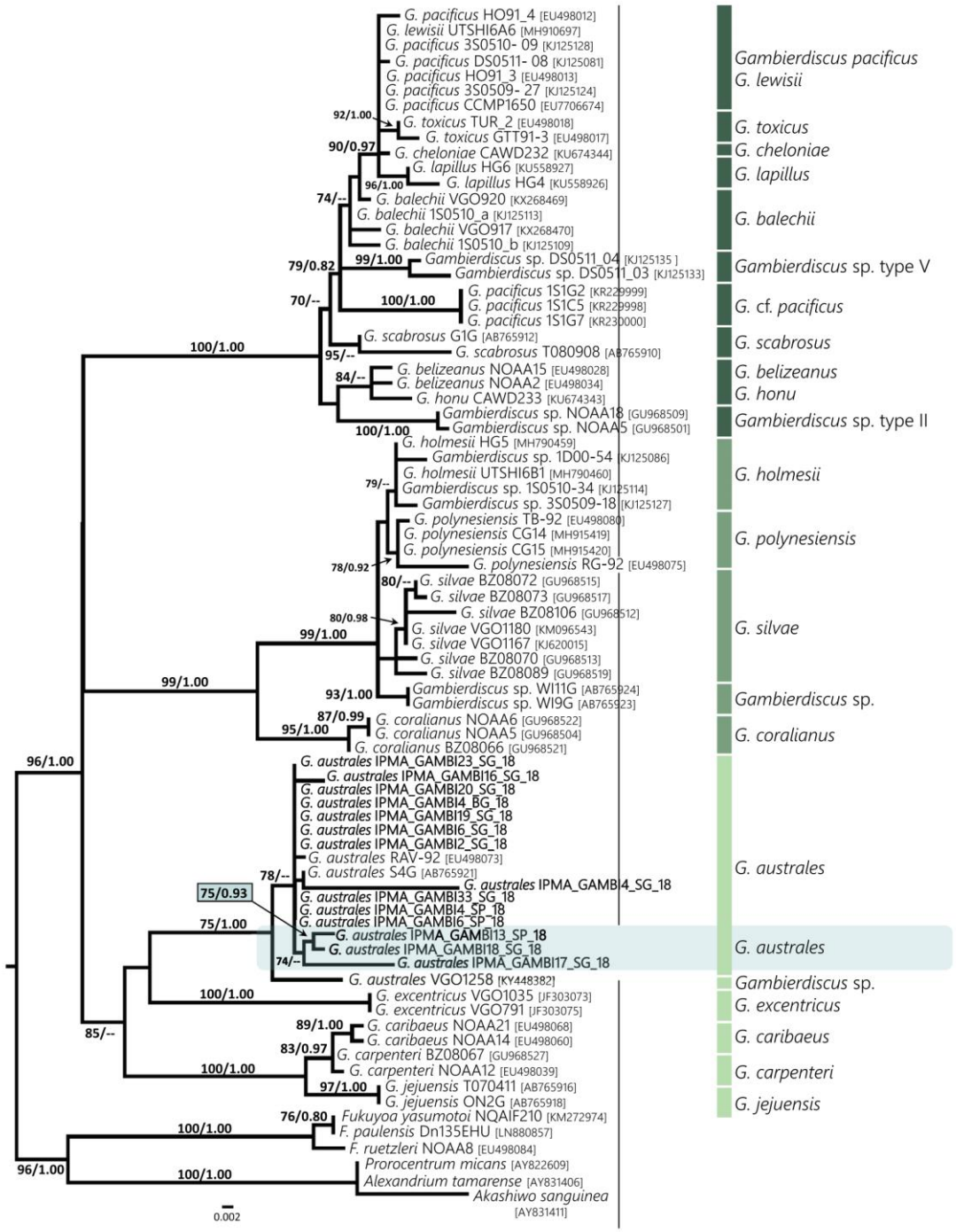

Fig. 4. Phylogenetic analysis of *Gambierdiscus* from Selvagens Islands based on D8–D10 LSU rDNA region. Node support values are bootstrap/Bayesian posterior probability obtained from Maximum likelihood and Bayesian inference analysis respectively, only the values with support above 70 are presented. The species sequenced in this study are in bold type. The sequences used to construct the phylogeny are presented with the species name, strain code and GenBank database identifier. Scale bar is substitutions per site. GenBank accession numbers from the sequences used in this study are displayed in Table 1.

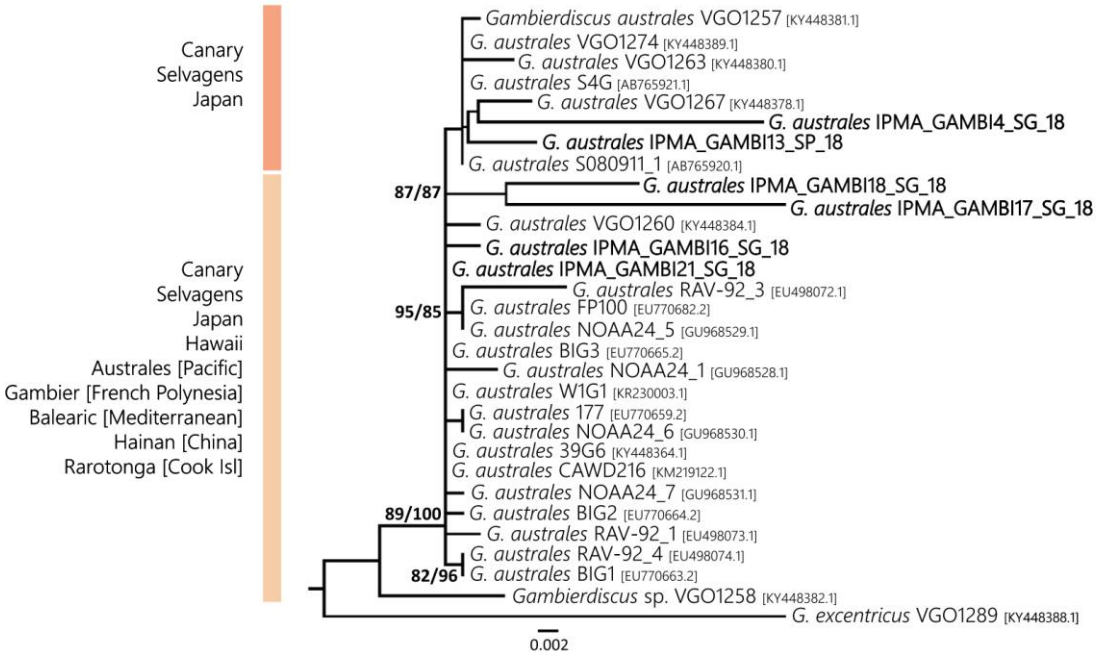

Fig. 5. Phylogenetic analysis of *Gambierdiscus australes* based on D8-D10 LSU rDNA region. Node support values are bootstrap/Bayesian posterior probability obtained from Maximum likelihood and Bayesian inference analysis respectively, only the values with support above 80 are presented. The species sequenced from this study are in bold type. GenBank accession numbers from these sequences are displayed in Table 1. The sequences used to construct the phylogeny are presented with the species name, strain code and GenBank database identifier. GenBank identifiers of redundant sequences are presented in Supplementary table S2. Scale bar is substitutions per site.

Table 3. Toxicity (fg CTX1B eq. cell^{-1}) of extracts of *Gambierdiscus* spp. from the Selvagem Islands, as evaluated by the Neuro-2A assay.

Strain	Species	Island	fg CTX1B eq. cell $^{-1}$
IPMA_GAMBI4_SP_18	*G. australes*	Selvagem Pequena	83
IPMA_GAMBI6_SP_18	*G. australes*	Selvagem Pequena	9.45
IPMA_GAMBI2_SG_18	*G. australes*	Selvagem Grande	9.21
IPMA_GAMBI4_BG_18	*G. australes*	Selvagem Grande	2.46

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary materials

Supplementary figure S1: Sea surface temperature measured in Cagarras Bay, Selvagem Grande (Madeira, Portugal) each day at 3:00 am and 4:00 pm between November 2016 and July 2018.

Supplementary figure S2: Meteorological data measured in Selvagem Grande in 2017–2019, a) air temperature (ºC), b) global radiation (kj m–2) and c) rain (mm). Box plot showing median, upper and lower quartiles, and 5th and 95th percentiles. Data from IPMA's automatic meteorological station.

Supplementary figure S3: Light microscopy micrographs of benthic dinoflagelates in Selvagem Pequena Island (Madeira, Portugal) at 200x magnification. A) Prorocentrum hoffmanianum, B) P. panamense, C) P. lima, D) P. concavum, E) P. emarginatum, F) Ostreopsis sp., G) Amphidinium sp., H) A. caterae, and I) P. rhathimum.Supplementary figure S4: Doseresponse curves of neuro-2a cells exposed to A) CTX1B standard, B) G. australes IPMA_GAMBI6_SP_18 extract, with (open circle) and without (filled circle) o/v treatment.

Supplementary table S1: Genetic distances (p distancemodel) of the D8-D10 region of the LSU rDNA within G.australes species (1–13) and between two close species (14–15).

Supplementary table S2: GeneBank® identifiers of redundant sequences presented in Figure 5.

Author contributions

L. Godinho: cell culturing, morphological description by microscopy, original concept, L. Soliño: cell culturing, sampling, original concept. C. Churro: molecular analysis. V. Timoteo: sampling; C. Santos: sampling. N. Gouveia: sampling. J. Diogène: cytotoxicity analysis, original concept. P.R. Costa: original concept, drafting and editing manuscript. All the authors were involved in the preparation of the manuscript and all have read and agreed with the final version.

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