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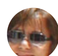
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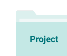
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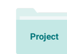
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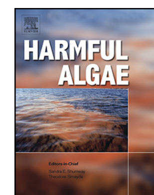

Morphology and phylogeny of *Prorocentrum caipirignum* sp. nov. (Dinophyceae), a new tropical toxic benthic dinoflagellate

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ABSTRACT

A new species of toxic benthic dinoflagellate is described based on laboratory cultures isolated from two locations from Brazil, Rio de Janeiro and Bahia. The morphology was studied with SEM and LM. Cells are elliptical in right thecal view and flat. They are 37–44 μm long and 29–36 μm wide. The right thecal plate has a V shaped indentation where six platelets can be identified. The thecal surface of both thecal plates is smooth and has round or kidney shaped and uniformly distributed pores except in the central area of the cell, and a line of marginal pores. Some cells present an elongated depression on the central area of the apical part of the right thecal plate. *Prorocentrum caipirignum* is similar to *Prorocentrum lima* in its morphology, but can be differentiated by the general cell shape, being elliptical while *P. lima* is ovoid. In the phylogenetic trees based on ITS and LSU rDNA sequences, the *P. caipirignum* clade appears close to the clades of *P. lima* and *Prorocentrum hoffmannianum.* The Brazilian strains of *P. caipirignum* formed a clade with strains from Cuba, Hainan Island and Malaysia and it is therefore likely that this new species has a broad tropical distribution. *Prorocentrum caipirignum* is a toxic species that produces okadaic acid and the fast acting toxin prorocentrolide.

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1. Introduction

More than one hundred species of the genus *Prorocentrum* Ehrenberg have been described and although some of them have been later identified as synonyms of other previously described species, 81 of these are considered valid (Guiry, 2017). It comprises both planktic and benthic species and among the latter, many of them produce toxins (Glibert et al., 2012). The production of biotoxins fostered many studies regarding the taxonomy and toxicology of these organisms. Until recently, the taxonomy of the genus *Prorocentrum* was based mainly on external morphology using criteria such as cell shape and size, thecal surface characteristics, intercalary band morphology, and architectural details of the periflagellar area. Some ultrastructural features including the presence or absence of trichocysts and mucocysts and the presence and organization of pyrenoids in the plastids were used as well (Hoppenrath et al., 2013). With the incorporation of molecular data to taxonomic studies, the taxonomy of all groups is under revision. As some of the morphological characters used for taxonomic descriptions of *Prorocentrum* species can be variable and subtle, the genetic analyses are important in *Prorocentrum* taxonomy in order to differentiate among cryptic species. This genus comprises two

Abbreviations: DTX1, dinophysistoxin 1; DTX2, dinophysistoxin 2; OA, okadaic acid; PTX, pectenotoxin 2.

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genetically distinct groups of species that were suggested to be considered as two separate genera (Boopathi et al., 2015). Most benthic species fall in a single clade lacking a spine that is present in most planktic species, and are symmetric in contrast to the asymmetric theca observed in most planktic species. The most conspicuous benthic *Prorocentrum* species is *P. lima* (Ehrenberg) Stein 1878 that has a broad geographic distribution and is considered a cosmopolitan species. Many cultured strains identified as *P. lima* have been analyzed and produce okadaic acid, dinophysistoxin 1 (DTX1) and analogues (Bravo et al., 2001; Nascimento et al., 2016), toxins that are responsible for incidents of diarrhetic shellfish poisoning (DSP) (Murakami et al., 1982). This species, originally described as *Cryptomonas lima* Ehrenberg, has been redescribed based on samples from its type locality, Naples, Italy (Nagahama and Fukuyo, 2005). An extensive study based on wild and cultured specimens of *P. lima* collected from different areas examined intraspecific variation in morphological and molecular genetic characters in order to identify species boundaries in *P. lima* (Nagahama et al., 2011). According to these authors, cell shape is variable in *P. lima* (Nagahama et al., 2011) and the species appears to contain cryptic diversity. Aligizaki et al. (2009) proposed the use of a "*P. lima* species complex" based on morphological variability and Hoppenrath et al. (2013) followed this proposal. Nagahama et al. (2011) emended the species description of *P. lima* and considered *Prorocentrum arenarium* as a synonym of *P. lima*. Zhang et al. (2015) characterized five *P. lima* morphotypes that were separated based on ITS and LSU rDNA sequences. More recently, Luo et al. (2017) renamed the strains comprised in two of these morphotypes (4 and 5) as *Prorocentrum* cf. *maculosum* and *P. maculosum*.

The discussion about the species concept is long and still very live. The Mayr's (1996) biological species concept is difficult to apply to protists as in most cases their complete life cycle is not known. When a "species complex" is mentioned, it is implicitly recognized that more than one species is present, not only one. The theoretical problem is to find a way to decide when in an evolutionary tree, two diverging branches can be considered two different species and when a gene flow barrier appears. Beyond the academic discussion about the species concept, to define species limits in toxin producing species is important from a practical point of view, as their toxin profiles can be different even among close species. Considering the *Alexandrium tamarense/catenella* species complex, in which some species produce toxins while others do not, the morphological criteria used to distinguish species did not match those based on genetics and toxin production. After the description of two new species and redescription of three of the species in the complex with emphasis on molecular-based (rDNA) classification by John et al. (2014) their toxicity became a consistent taxonomic character among those *Alexandrium* species. Another example is the genus *Pseudo-nitzschia* which comprises several species-complexes with cryptic and pseudo-cryptic species (Percopo et al., 2016; Trainer et al., 2012), that need the help of molecular tools to be identified.

In the current study, based on samples taken from two localities from the Brazilian coast, a new toxic benthic *Prorocentrum* species that belongs to the genetic group of benthic symmetric species

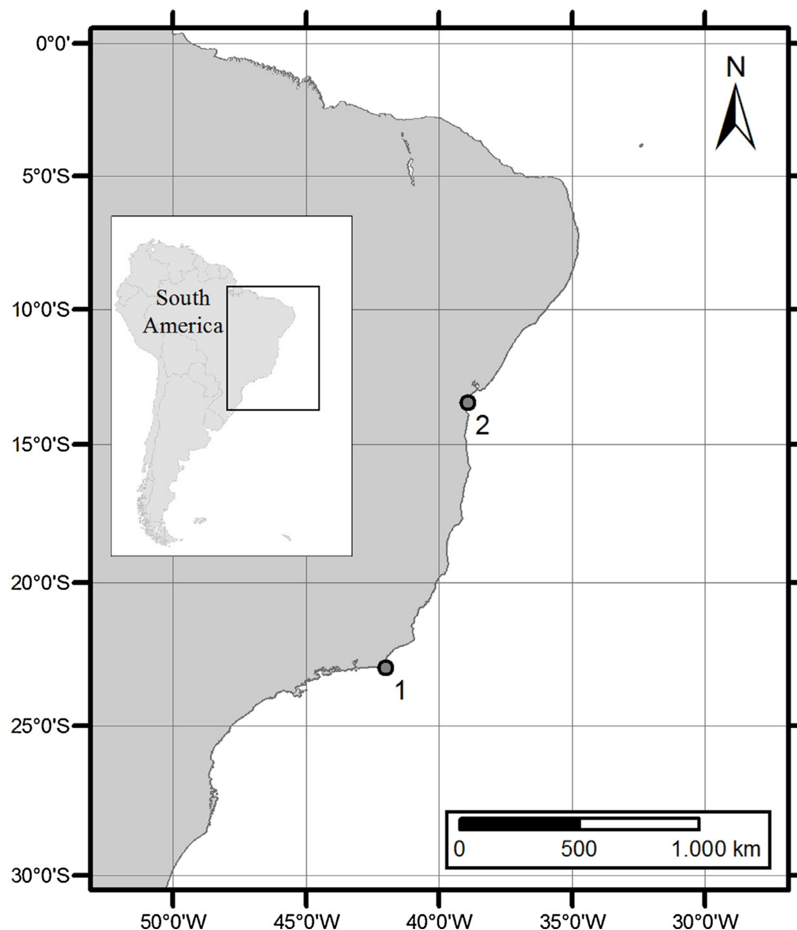


Fig. 1. Map of South America showing the two locations where *P. caipirignum* was isolated from. 1- Arraial do Cabo, Rio de Janeiro and 2 – Garapuá, Ilha de Tinharé, Cairu, Bahia, Brazil.

(Boopathi et al., 2015) is described based on morphology, genetics and toxicity, with the name *P. caipirignum*. Strains of this new species were previously considered as *P. lima, P. arenarium* or as *P. maculosum* by other authors.

2. Materials and methods

2.1. Isolation of strains and establishing cultures

The *P. caipirignum* strain LCA-B4 was established in March 2007 from the macroalgae *Sargassum vulgare* C. Agardh collected from Arraial do Cabo (23°00′03″ S, 42°00′22″ W), Rio de Janeiro (Fig. 1). Specimens of *S. vulgare* were placed in sealable plastic bags and were vigorously shaken for 2 min to detach the associated epiphytic cells. Live cells of *Prorocentrum* were isolated from the epiphytic suspension using a micropipette, and were sequentially transferred through four to five drops of sterile and filtered (glass-fiber filter, Millipore AP-40, Millipore Brazil) local seawater. After each transfer, the drop was examined to ensure a single cell was present. After the final transfer, each isolated cell was placed into a separate well of a sterile 96-well tissue culture plate with 90 μL of L2 culture medium (Guillard, 1995) modified by omitting silicate, nickel, vanadium and chromium and prepared with local seawater which had been filtered (glass-fiber filter, Millipore AP-40, Millipore Brazil) and autoclaved. When sufficient cell density was achieved, cells were transferred to a separate well of a sterile 6-well tissue culture plate containing L2 medium and were eventually transferred to 250 mL glass Erlenmeyer flasks.

All stock cultures were maintained in a temperature controlled cabinet at 24 ± 2 °C, with a light/dark cycle of 12 h:12 h and a photon flux density of 60 $\mu mol\ m^{-2}\ s^{-1}$ provided by cool-white fluorescent tubes. Photosynthetically active radiation was measured with a QSL-100 quantum sensor (Biospherical Instruments, San Diego, CA, USA).

The *P. caipirignum* strain UFBA064 was established from a sample obtained from a mixture of macroalgae species: *Tricleocarpa fragilis* (Linnaeus) Huisman & R.A.Townsend, *Tricleocarpa cilindrica* (J.Ellis & Solander) Huisman & Borowitzka, *Palisada perforate* (Bory) K.W.Nam, *Halimeda opuntia* (Linnaeus) J.V. Lamouroux, *Caulerpa racemosa* (Forsskål) J.Agardh, *Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier in Castagne and *Amphiroa fragilissima* (Linnaeus) J.V.Lamouroux, collected in May 2014 at Garapuá, Tinharé island, Cairu, Bahia (13°29′17.35″S, 38°54′22.7″W) (Fig. 1). Cells were dislodged from their substrates using the same method described above. Isolated cells were kept initially in sterile Guillard's f/2 marine water enrichment solution (Sigma-Aldrich, St. Louis, MO, USA) in one well of a 20-well tissue culture plate, then were transferred to 50 mL glass Erlenmeyer flasks with K/2 medium (Keller et al., 1987) without silicate. Cells were maintained in a temperature controlled cabinet at 25 ± 2 °C, with a light/dark cycle of 12 h:12 h and a photon flux density of 60 $\mu mol\ m^{-2}\ s^{-1}$ provided by cool-white fluorescent tubes.

2.2. Light microscopy

Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with phase contrast, differential interference contrast and epifluorescence with an UV lamp and with UV and blue excitation filters. The cultured cells were observed alive or preserved with formalin. To study the pore pattern at the thecal plate surface, cells were stained with Fluorescent Brightener 28 (Sigma-Aldrich, St Louis, MO, USA) following a modified technique (Fritz and Triemer, 1985). Cells were dissected using drops of diluted bleach added to the slides and gently pressing the cover slip over them under observation at the microscope. If necessary, deionized water (dH_2O) was added to the slide to replace evaporated volume. Images were collected using an Axiocam HRc (Carl Zeiss, Jena, Germany) camera and Zen image acquisition and analysis software (Zeiss, Germany). When the depth of field was insufficient to capture the whole object, a series of pictures was taken at different focal planes, and these pictures were merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).

2.3. Scanning electron microscopy

For scanning electron microscopy (SEM) observations of strain LCA-B4, cells were preserved with 2% paraformaldehyde for 2 h and were then rinsed three times with seawater salinity 28 and two times with 0.1 M sodium cacodylate buffer. The cells were adhered to a poly-L-lysine coated glass coverslip and then dehydrated through an ethanol series (10, 30, 50, 70, 80, 90, 100%) and subsequently dried using a critical point dryer EM CPD300 (Leica Microsystems, Vienna, Austria). The glass coverslip was sputter-coated with gold. Observations were made using a FEI Quanta 450F, HV 5.00 kv, WD 9.4 mm (FEI Company, Hillsboro, OR, USA).

2.4. Molecular analyses: PCR amplification and DNA sequencing

DNA extraction from *P. caipirignum* strain LCA-B4 was performed using a modified guanidinium isothiocyanate protocol (Chomczynski and Sacchi, 2006) as described by Alves-de-Souza et al. (2011). The ITS-1/5.8S/ITS-2 regions of strain LCA-B4 was amplified with ITSA/ITSB primers (5′-CAA GCT TCT AGA TCG TAA CAA GGH TCC GTA GGT-3′/5′-CTG CAG TCG ACA KAT GCT TAA RTT CAG CRG G-3′). The PCR mix (15 μL final volume) contained 1–6 μL of the DNA extract, 330 μM of each deoxynucleoside triphosphate (dNTP), 2.5 mM of $MgCl_2$, 1.25 units of GoTaq® DNA polymerase (Promega Corporation), 0.17 μM of both primers, 1 x reaction buffer (Promega Corporation). ITS amplification was performed with an initial denaturating step at 95 °C for 5 min, 35 cycles at 95 °C for 1 min, annealing at 55 °C for 45 s, extension at 72 °C for 1 min 15 s, with a final extension step at 72 °C for 7 min. PCR products were cloned using the TOPO TA Cloning® kit (Invitrogen) according to manufacturer's recommendations, and selected clones were amplified by PCR following the protocol described above. PCR products were purified using the ExoSAP-IT kit (USB) following the manufacturer's recommendations and directly sequenced on an ABI Prism 3100 automatic sequencer (Applied Biosystems). ITS sequences obtained in this study (593 nt) were deposited in GenBank (for Accession numbers see Fig. 6).

Exponentially growing cells of *Prorocentrum caipirignum* UFBA064 (1 mL) were harvested by centrifugation (15800*g*; 2 min) using an Eppendorf 5424R centrifuge (Eppendorf AG, New York, USA) and the cellular pellets were rinsed in 1 mL distilled water, centrifuged again and the supernatant discarded. The concentrate was transferred to 200 μL microtubes and frozen at −20 °C until analysis. DNA was extracted using two methods. A modified Chelex procedure (Richlen and Barber, 2005) was used for ITS and 100 μL of 10% Chelex100 (Bio-Rad, Hercules, CA, USA) in dH_2O was added to the cell pellets and samples were transferred to 200 μL tubes. The samples were heated to 95 °C in a Surecycler 8800 thermocycler (Agilent Technologies, Santa Clara, CA, USA) for 10 min and then vortexed. The heating and vortex steps were done twice. Samples were centrifuged (15800*g*; 2 min) and the supernatants transferred to clean 200 μL tubes avoiding to carryover the Chelex beads. Genomic DNA was quantified and checked for its purity in a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA). For LSU analyses, DNA was extracted using NZY Plant/Fungi gDNA Isolation kit (NZY Tech), following

manufacturer's instructions, proceeded by lysing the cells with glass beads.

The D1/D2 domains of the LSUrRNA gene of strain UFBA064 were amplified using the pair of primers D1R/D2C (5′-ACCCGCTGAATTTAAGCATA-3′/5′-ACGAACGATTTGCACGTCAG-3′; Lenaers et al., 1989). The ITS-1/5.8S/ITS-2 regions were amplified with ITSF01/PERK-ITS-AS (5′-GAGGAAGGAGAAGTCGTAACAAGG-3′/5′-GCTTACTTATATGCTTAAATTCAG-3′; Kotob et al., 1999). The amplification reaction mixtures (25 μL) contained 1 x PCR buffer, 2 mM $MgCl_2$, 0.5 pmol of each primer, 2 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, California, USA) and 2 μL of DNA extracts. DNA was amplified in an Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA) following the conditions detailed in Lenaers et al. (1989). ITS amplification of UFBA064 followed an initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 2 min, extension at 72 °C for 3 min, and a final extension cycle at 72 °C for 10 min. 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 C.A.C.T.I. sequencing facilities (Centro de Apoyo Científico Tecnolóxico á Investigación, Universidade de Vigo, Spain). The ITS and LSU rDNA sequences obtained in this study (666 and 602 nt, respectively) were deposited in GenBank (for Accession numbers see Figs. 6 and 7).

2.5. Phylogenetic analyses

Amplified sequences of strains LCA-B4 and UFBA064 were inspected and aligned using CLUSTALW multiple alignment in Bioedit (Hall, 1999). The final LSU and ITS alignments used for phylogenetic calculations included 550 and 630 positions, respectively. *Adenoides eludens* and *Heterocapsa triquetra* sequences were used as outgroups for the LSU and ITS analyses. The phylogenetic relationships were determined using Maximum Likelihood (ML) phylogenetic analyses with MEGA7 (Kumar et al., 2016), and Bayesian phylogenetic inference with Mr. Bayes v.3.2.4 (Huelsenbeck and Ronquist, 2001). In the case of ML phylogeny, model selection was performed using the best model search tool available on MEGA7. The model selected for LSU and ITS analyses was Kimura-2 parameter (Kimura, 1980) and Tamura-Nei models (Tamura and Nei, 1993), with gamma shape parameter 1.17 and 0.45, respectively. Bootstrap values were estimated from 1000 replicates. In the case of Bayesian analyses the substitution models were obtained by sampling across the entire GTR model space following the procedure described in Mr. Bayes v3.2 manual. The program parameters were statefreqpr = dirichlet (1,1,1,1), nst = mixed, rates = gamma. The phylogenetic analyses involved two parallel analyses, each with four chains. Starting trees for each chain were selected randomly using the default values for the Mr. Bayes program. The corresponding number of unique site patterns was 280 and 323 in LSU and ITS analyses. The number of generations used in these analyses was 1.000,000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization ("burn-in" phase). All final split frequencies were < 0.025. The phylogenetic trees were represented using the ML results and bootstrap values from ML, and posterior probability values from Bayesian analyses.

2.6. Toxin analysis

Two *Prorocentrum caipirignum* cultures of strain LCA-B4 (50 and 35 mL in modified L2 medium in 125 mL Erlenmeyer flasks) were maintained as described in section 2.1. After 14 days of cultivation, the volume of each culture was measured and transferred to two centrifuge tubes. A sub-sample of 3 mL was removed from each centrifuge tube and preserved with neutral Lugol's iodine solution for cell enumeration using a Sedgewick Rafter counting chamber. Cultures were harvested by centrifugation for 10 min (1600*g*; 10 °C). Immediately after decanting the supernatant, the cell-free media was removed and one tube from each culture containing cell pellets were immersed in a bath of boiling water for 5 min to denature enzymes and preserve the native profile of 'free' OA/DTXs. The second tube from each culture containing cell pellets was kept at room temperature (~25 °C), allowing the conversion of OA and DTXs derivatives to their respective parent toxins. Cell pellets were kept at −80 °C prior to toxin extraction and analysis.

Extraction of toxins from boiled (heat-treated) and non-boiled cell pellets involved vortex mixing (3 min) the *P. caipirignum* LCA-B4 cell pellets with 1 mL of 90% aqueous methanol for 3 min. Extracts were centrifuged (as described above) and cell pellets were extracted again. Combined extracts were evaporated to near dryness under a gentle stream of oxygen-free nitrogen and reconstituted in 0.5 mL methanol. Extracts were then transferred to 2 mL autosampler vials and stored at −20 °C prior to analysis.

Extracts of the *P. caipirignum* LCA-B4 cells were analysed for OA, DTX1 and DTX2 toxins by liquid chromatography coupled to quadrupole mass spectrometry [LC–MS/MS; Agilent 1100 series LC (Agilent Ltd., Manchester, United Kingdom) and Waters Quattro Micro (Waters Ltd., Manchester, UK)]. Toxin analytes were separated using a Waters XBridge column (150 × 2.1 mm; 3.5 μm; Waters Ltd.) and an alkaline (pH 11; flow rate of 0.35 mL min^{-1}) mobile phase gradient (Gerssen et al., 2009). Analytes were ionised by electrospray (negative) ionisation (ESI) and detected by multiple reaction monitoring (MRM) using two transitions per toxin [for OA and DTX2 (803.4 > 113.0 and >255.3); for DTX1 (817.4 > 113.0 and >255.2)]. Toxin quantities were determined by external calibrations and direct comparisons of signal responses generated from a series of OA and DTX calibration reference standards (Institute for Marine Biosciences, National Research Council Canada, Halifax, Nova Scotia, Canada). Deploying both positive and negative ionisation modes, and separately full scan (m/z 100 to 1100; mobile phase flow 0.30 mL min^{-1}) and selected ion recording (SIR; cone voltage 50 V; mobile phase flow rate 0.45 mL min^{-1}), extracts from strain LCA-B4 were also analysed to qualitatively assess the presence of the prorocentrolide toxins.

A culture volume of 100 mL of the *P. caipirignum* strain UFBA064 (at exponential phase) was processed for toxin analyses. A sub-sample of 1.5 mL was removed from the culture and preserved with acidic Lugol iodine solution for cell enumeration using a Sedgewick Rafter counting chamber. All of the culture volume was subsequently filtered through glass microfiber filters of 47 mm diameter (MFV4, C de-Parmer Instrument, Filter-Lab, USA). Cells contained in the filters were re-suspended in methanol and sonicated using the sonication probe 4710 of the Ultrasonic Homogenizer (Cole-Palmer, Chicago, IL. USA). The suspension was then centrifuged at 5411*g* for 10 min at 10 °C. The supernatant was removed and the cell pellet was extracted again with methanol following the same procedure. Both supernatants were combined and the total volume was adjusted to 3 mL with methanol and stored at −20 °C until the analysis by LC-HRMS.

Mass spectrometry analyses were performed using a Thermo Scientific Dionex LC coupled to an Exactive mass spectrometer, equipped with an Orbitrap mass analyzer. The instrument was mass calibrated for positive and negative modes, and the capillary and tube lens voltages were also optimized, using the automated script within the Exactive acquisition software in both cases. HRMS experiments were carried out in positive and negative modes, without and with all ion fragmentation (AIF) (HCD 35 eV). The

mass range for both of them, full MS and AIF mode, ranged from 100 to 1500 m/z at a resolving power 70,000.

The following conditions were used: a GeminiNX C18 column, 3.5 μm 50 × 2.10 mm (Phenomenex) was kept at 40 °C and eluted at 0.4 mL min^{-1} with 0.05% ammonia (eluent A) and 95:5 (acetonitrile/0.05% ammonia) (eluent B). The following gradient elution was used: 25% B at t = 0, 25% B at t = 1.5, 95% B at t = 7.5, 95% B at t = 9.5, 25% B at t = 12.5, and hold for 1.5 min. For toxins identification an analytical standard mix solution in methanol containing 20 ng mL^{-1} of each of the following toxins: OA, DTX2, DTX1 and PTX2 was used. The presence of prorocentrolide, belizeanolide, belizeanic acid and a series of OA-esters were investigated (Table 1). Since no reference material neither standards were available for OA congeners, those peaks were identified in the chromatogram by the exact mass of diagnostic, the isotope ion pattern and the mass accurary (± 5 ppm extraction window).

3. Results

3.1. *Prorocentrum caipirignum* S. Fraga, M. Menezes et S. Nascimento sp. nov

Description: Cells of *Prorocentrum caipirignum* are elliptical, 37–44 μm long and 29–36 μm wide. Both the thecal surface and intercalary bands are smooth. The thecal plates have a line of marginal pores and scattered pores except in the center which is devoid of pores. The pores can be circular but in most cases are elongated or kidney-shaped. The periflagellar area has a V shape on the right thecal plate without or with a low collar on the left thecal plate. It is composed of 6 platelets that leave place for a flagellar and an accessory pore and has no spines. The nucleus is posterior. Cells are photosynthetic and have branched and reticulated chloroplasts that surround two central pyrenoids.

Holotype: Fig. 3A from strain LCA-B4 conserved on SEM-stub (designation R 229577) deposited at the herbarium of the Museu Nacional, UFRJ (Brazil).

Additional information: The culture of strain LCA-B4 used for the holotype was lost before this work was finished. Strain UFBA064 can be used as an alternative of strain LCA-B4. Strain UFBA064 is maintained at Museu Nacional, UFRJ (Brazil) and at the Culture Collection of Harmful Microalgae of Centro Oceanográfico de Vigo (CCVIEO).

Molecular characterization: strain LCA-B4 (Acc. No.: KJ960192) and strain UFBA064 (Acc. Nos.: KY039499 and KY039500).

Etymology: The epithet *caipirignum* makes reference to "caipirinha", a popular cocktail typical and original from Brazil but also consumed in many other countries of the world. Although *P. caipirignum* is described from Brazil, it is also present in other countries.

Type locality: Arraial do Cabo (23°00′03″ S, 42°00′22″ W), Rio de Janeiro, Brazil (Fig. 1).

Habitat: *Prorocentrum caipirignum* grows epiphytically on macroalgae in tropical marine areas.

3.2. Morphology

Cells of *Prorocentrum caipirignum* are photosynthetic and have branched and reticulated chloroplasts (Fig. 2A) that surround two central pyrenoids that can be observed by light microscopy changing focus (Fig. 2B). The nucleus is posterior (Fig. 2B). Cells are elliptical, 37–44 μm long and 29–36 μm wide (n = 20) (Figs. 2 and 3). The length/width ratio ranges between 1.17 and 1.37. The thecal plates are flat with smooth thecal surface and intercalary bands (Figs. 3 and 4) and the right thecal plate has frequently a longitudinal depression in the anterior half (Figs. 3 A and 4 A). Both thecal plates have a line of 56 ± 3 (n = 12) marginal pores and 69 ± 7 (n = 12) scattered pores that in many cases can be almost ordered in three concentric lines parallel to the marginal pores line, except in the center which is devoid of pores (Fig. 3). All pores are large (0.50–0.90 μm), can be circular but in most cases are elongated or kidney-shaped (Figs. 3 and 4). The anterior margin of the left thecal plate can be convex, flat or slightly concave (Fig. 3D–F).

The periflagellar area has a V shape on the right thecal plate without or with a low collar on the left thecal plate (Fig. 5). No spines, thick flange, platelet lists or protrusions were observed. It is composed of 6 platelets which have been labeled according to Hoppenrath et al. (2013) scheme (Fig. 5). Each platelet has a single depression that may look like a hole. It has a big oval flagellar pore and a smaller accessory pore (Fig. 5). Platelet 1 is three-lobed in the external part and has an extension below one side of platelet 2 contributing to the left side of the accessory pore, although it is possible that the lower part of platelet 1 corresponds to platelet 7 (Fig. 5C). Platelet 2 is pentagonal and has an extension towards the right thecal plate that separates the accessory pore from the flagellar pore. Platelet 3 is trapezoidal. Platelet 4 is elongated and contacts the two thecal plates and platelets 3 and 5. Platelet 5 is elongated and curved forming most of the side of the flagellar pore

Table 1
Molecular formula, m/z ions in positive and negative ion modes and retention times (RTs) of toxins analyzed in *Prorocentrum caipirignum* strain UFBA064. HRMS data are detailed in the detected compounds. Assignment of molecular formula, relative double bonds (RDB) equivalents and error (ppm) is showed.

Compound	Molecular formula	m/z			RT(min)	RDB	Δ ppm	UFBA064
		pos [M+Na]$^+$	pos [M+H]$^+$	neg [M-H]$^-$				
OA	$C_{44}H_{68}O_{13}$	827,4536		803,4592	3,7	10,5	−1829	+
DTX1	$C_{45}H_{70}O_{13}$							ND
DTX2	$C_{44}H_{68}O_{13}$							ND
Prorocentrolide	$C_{56}H_{85}NO_{13}$		980,6081	978,5944	4,85	14,5	−0,987	+
Belizeanolide	$C_{81}H_{132}O_{20}$							ND
Belizeanic acid	$C_{44}H_{72}O_{14}$	847,4821			8,47	8,5	0,793	Traces
Methylokadaate	$C_{45}H_{70}O_{13}$	841,4693		817,4744	7,66	10,5	−2214	+
Norokadanone	$C_{43}H_{66}O_{11}$							ND
Compound 5	$C_{48}H_{74}O_{14}$							ND
Compound 6	$C_{50}H_{76}O_{14}$							ND
Compounds 7/8	$C_{53}H_{82}O_{14}$	965,5574			8,09	12,5	−2256	+
Compound 9	$C_{53}H_{82}O_{15}$	981,5521			10,23	12,5	−2,54	+
Compound 10	$C_{53}H_{82}O_{16}$							ND
DTX6	$C_{51}H_{76}O_{14}$							ND
Compound 12/OA-D8-diol-ester	$C_{52}H_{80}O_{14}$	951,5422			7,76	12,5	−1921	+
Compound 13	$C_{54}H_{82}O_{14}$							ND

ND: not detected. Compounds 5,6,7,8,9,12 and 13 are OA derivatives described in Paz et al. (2007).

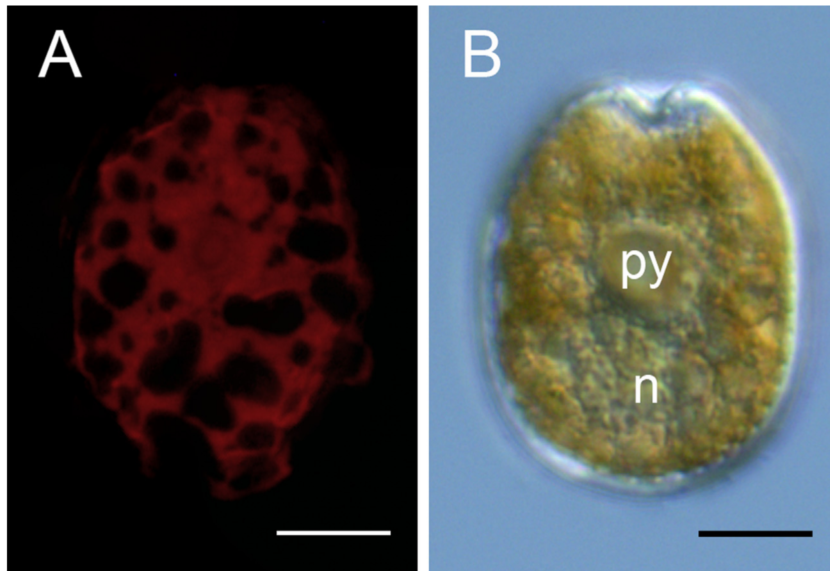


Fig. 2. Light microscopy images of *Prorocentrum caipirignum* (Strain UFB064). (A) Epifluorescence image of chloroplasts. (B) Differential interference contrast (DIC) microscopy of a live cell. Pyrenoids (py) and nucleus (n). Scale bars: 10 μm.

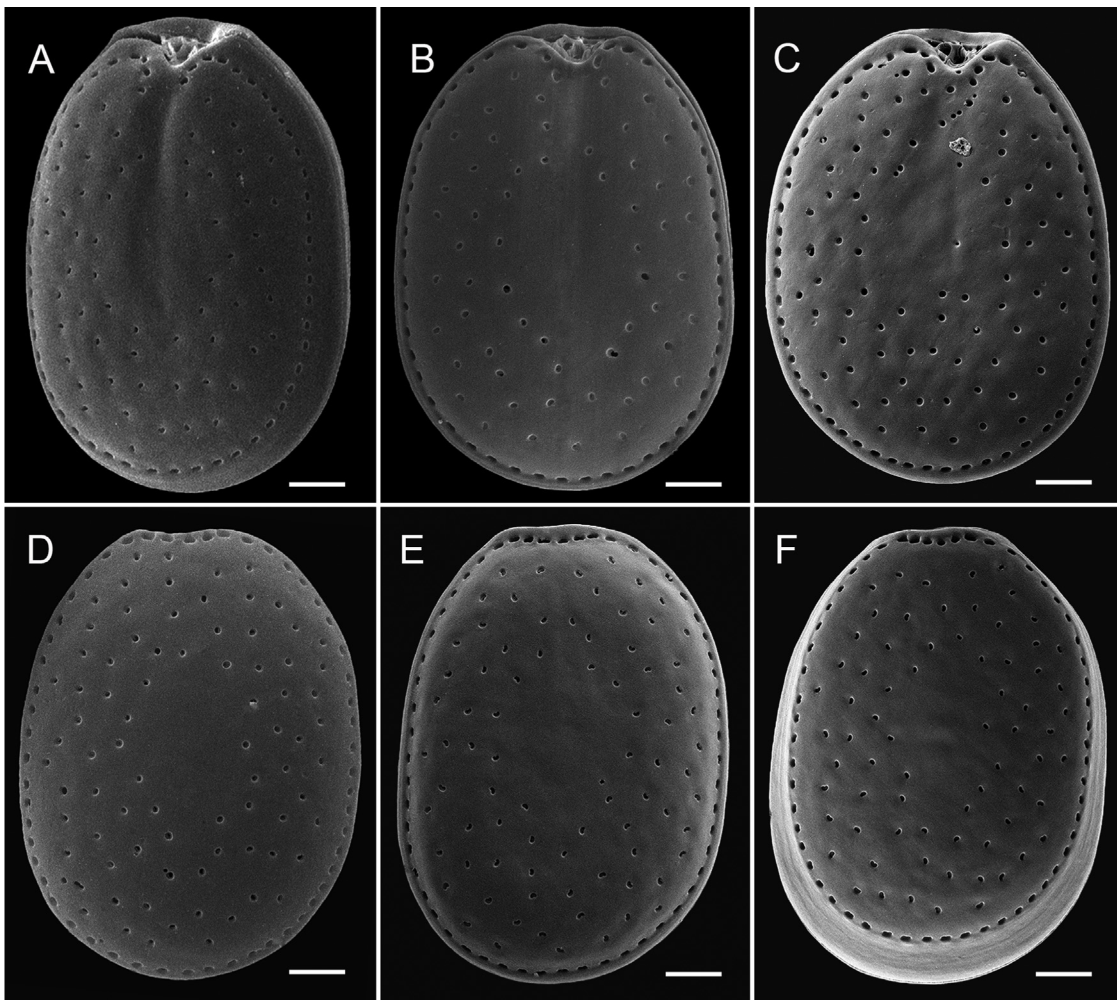


Fig. 3. Scanning electron microscopy (SEM) images of *Prorocentrum caipirignum* cells (Strain LCA-B4). (A-C) Right thecal plate view. (D-F) Left thecal plate view. Scale bars: 5 μm.

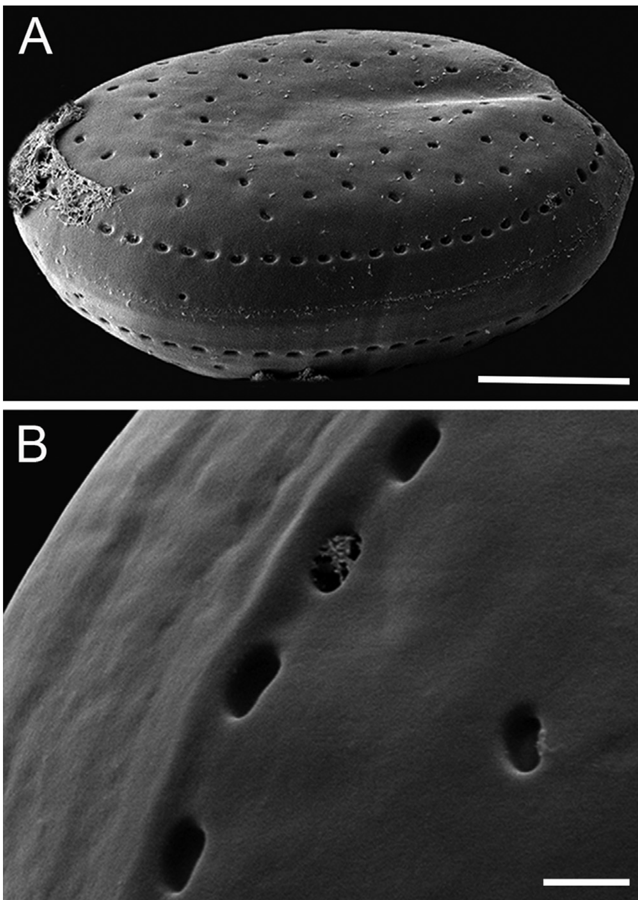


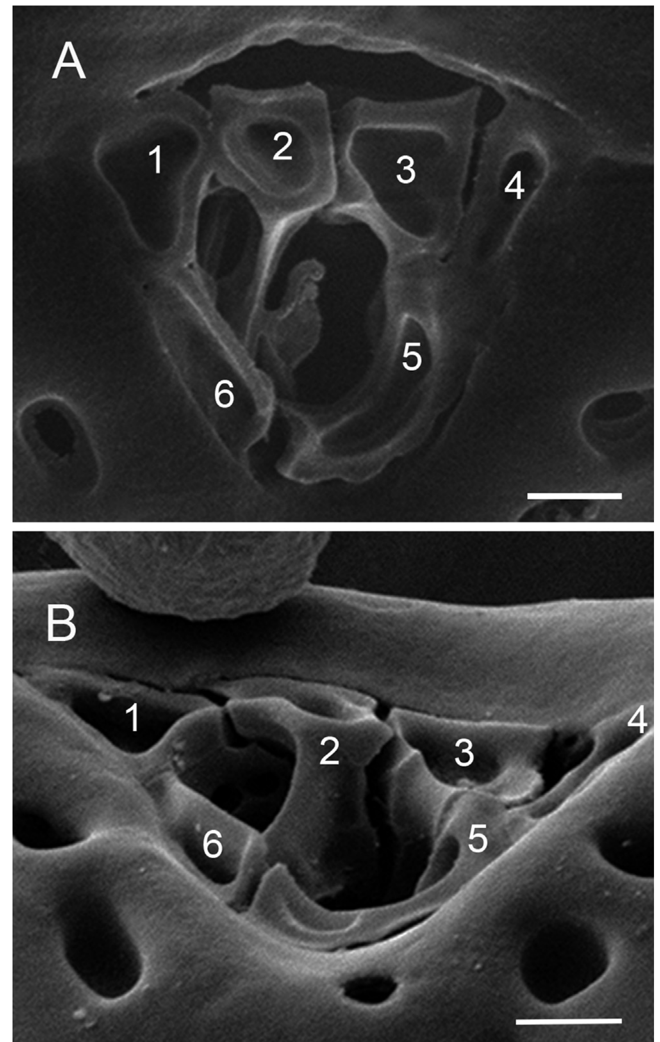
Fig. 4. SEM images of *Prorocentrum caipirignum* (Strain LCA-B4). (A) Oblique view of right thecal plate and intercalary band. (B). Detail of intercalary band and marginal pores. Scale bars: (a) 10 μm, (b) 1 μm.

opposed to the accessory pore. Platelet 6 is elongated and forms the right side of the accessory pore (Fig. 5).

3.3. Phylogeny

The phylogenetic results for *Prorocentrum* strains used in the present study are shown in Figs. 6 and 7. Both LSU rRNA gene and ITS phylogenies placed LCA-B4 and UFBA064 strains in a group (termed hereafter *P. caipirignum*) closely related to the *P. hoffmannianum* species complex and to the clade containing strains AS4F8, DS4D9 and DS4G4 (*P. lima* morphotype 5 from Zhang et al., 2015 or *P. maculosum* according to Luo et al., 2017). Support for these groups was robust in the ITS tree (Fig. 6). In the LSU phylogeny (Fig. 7), *P. caipirignum* clade included strain UFBA064 together with strains PMHV-1 and K-0625 (labeled as *P. maculosum* and *P. arenarium*, respectively), strains SE10, TIO11, TIO102 and TIO138 (labeled as *P. lima* morphotype 4 according to Zhang et al., 2015 and as *P.* cf. *maculosum* in Luo et al., 2017). These strains displayed only 0–2 different nt, whereas 4–5 nt were found relative to *P. lima* morphotype 5, and 9–16 nt relative to sequences in the *P. hoffmannianum* clade. The differences between *P. caipirignum* and *P. hoffmannianum* clades in the LSU alignment (in terms of genetic *p*-distance) ranged from 1.3-1.9%, whereas these values were slightly lower (0.8-1.0%) in relation to *P. lima* morphotype 5 of Zhang et al. (2015).

ITS phylogeny clearly separated a *P. caipirignum* clade consisting of seven strains: UFBA064 and LCA-B4, *P. maculosum* PMHV-1, TIO102, TIO138, TIO139 and *P. lima* SE10 (Fig. 6). The number of nt differences in the ITS alignment varied from 0 to 4 among



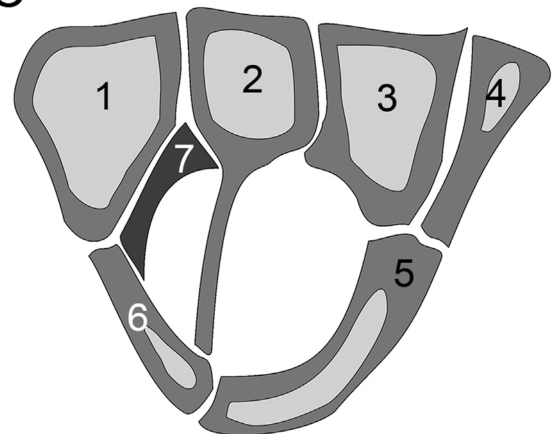


Fig. 5. *Prorocentrum caipirignum* periflagellar area. (A-B) SEM images of strain LCA-B4. (C) Schematic view of periflagellar platelets pattern. Scale bars: 1 μm.

P. caipirignum strains, whereas 54–63 different nt were found between *P. caipirignum* relative to *P. hoffmannianum* clade and 54–58 nt in relation to *P. lima* morphotype 5 clade (sensu Zhang et al., 2015). Genetic *p*-distances between members of

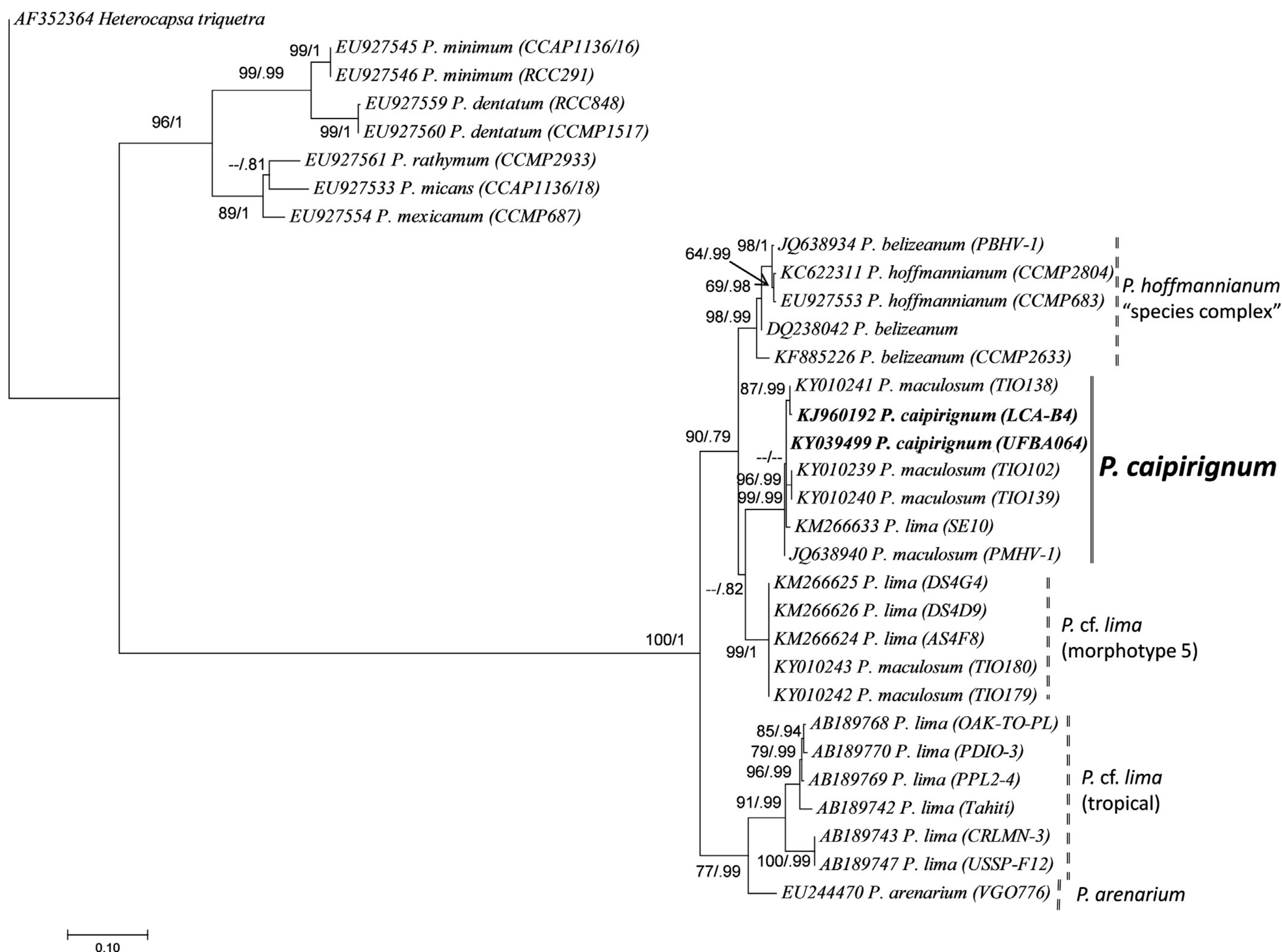


Fig. 6. ITS phylogeny (ITS-1/5.8S/ITS-2) showing the relationship between *Prorocentrum caipirignum* strains (in bold) and other *Prorocentrum* species. Supports at internal nodes are bootstrap values obtained by Maximum Likelihood method and posterior probability values (Bayesian analyses). Hyphens indicate bootstrap values <60.

P. caipirignum and *P. hoffmannianum* clades ranged from 6.8-9.1%, with slightly lower values (7–7.8%) for *P. caipirignum vs P. lima* morphotype 5.

3.4. Toxins

Using LC–MS/MS and deploying multiple reaction monitoring (MRM) it was apparent that only OA was detected in strain LCA-B4. The chromatographic retention time (8.3 min) of the two MRM transition ions (803.4 > 113.0 and >255.3) of certified OA reference standard (Fig. 8A and B) matched that of the same MRMs of the peak observed in the *P. caipirignum* LCA-B4 sample extracts (Fig. 8C and D) and confirmed the identity of this toxin. Comparing retention times for the reference standards, DTX2 (8.6 min; Fig. 8A and B) and DTX1 (9.3 min; Fig. 8E and F) with the respective MRM channels for these toxins in *P. caipirignum* LCA-B4 extracts (Fig. 8C and D and G and H), neither DTX1 or DTX2 toxins were evident. The total OA content (from non-boiled samples) quantified by comparison with certified reference standards was 20 pg cell^{-1} (average value of two cultures of strain LCA-B4). Where inhibition of the hydrolytic conversion of OA and DTX water soluble derivatives (e.g., DTX4s) was performed via boiling the cell pellets prior to extraction, 'free' OA concentration was approximately ten times less, and an average of 2 pg cell^{-1} was determined.

Extracts of *P. caipirignum* LCA-B4 were also analysed using positive and negative electrospray ionisation and full scan MS mode. In both ionisation modes and using a slightly slower LC flow rate of 0.3 mL min^{-1}, two peaks were present at 8.6 and 9.3 mins (Fig. 9A and B). Mass spectra of the first eluting peak represented OA (Fig. 10). In positive ionisation, the sodiated OA ion $[M+Na]^+$ m/z 827 as well as a series of $[M+H–_nH_2O]^+$ ions (e.g., m/z 787, 769, 751, 733, 715, 679) were observed (Fig. 10A). The OA toxin was further confirmed during electrospray negative ionisation and by the presence of the dominant deprotonated $[M–H]^-$ ion (m/z 803) (Fig. 10B). Based on the reported molecular formula of prorocentrolide being $C_{56}H_{85}NO_{13}$ (MH $+m/z$ 980.6168) and from the detection of m/z 981 and m/z 979 from full scan analyses (Fig. 11A and B) a tentative identification of the nitrogenous macrolide, prorocentrolide toxin was made. Further LC (flow rate 0.45 mL min^{-1}) and selected ion recording (SIR) analyses using both positive (m/z 980.5; $[M+H]^+$) and negative (m/z 978.5; $[M-H]^-$) ionisation modes revealled the tentative presence of the toxin which eluted at 8.6 min (Fig. 12).

For the analysis of strain UFBA064, the four toxin standards, OA, DTX1, DTX2 and PTX2 were baseline separated in just under 8 min (Fig. 13). Presence of OA was unambiguously confirmed by LC-HRMS in positive and negative mode and the estimated OA concentration was 0.15 pg cell^{-1}. DTX2, DTX1 and PTX2 were not detected. Prorocentrolide, methylokadaate, compounds 7/8, 9 and 12 (named OA-D8-diol-ester) were found (Fig. 14). Since paired compounds 7 and 8 have identical molecular weight and no

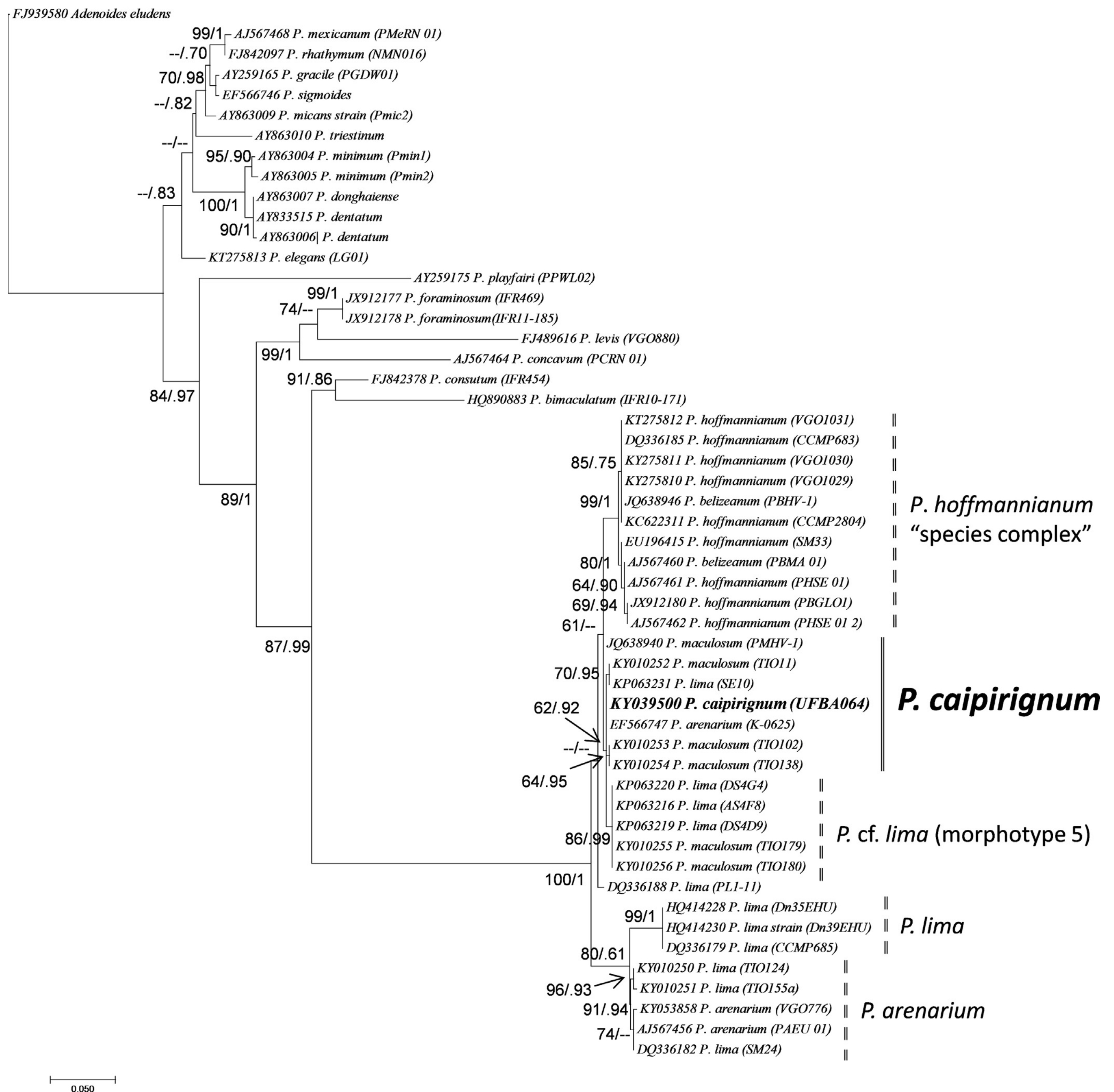


Fig. 7. LSU phylogeny (D1/D2 region) showing the relationship between *Prorocentrum caipirignum* (UFBA064, in bold) and other *Prorocentrum* species. Supports at internal nodes are bootstrap values obtained by Maximum Likelihood method and posterior probability values (Bayesian analyses). Hyphens indicate bootstrap values <60.

standards are available, it was not possible to confirm the identity of those compounds.

3.5. Ecology

Prorocentrum caipirignum was found epiphytically on macroalgae in an assemblage that included *Prorocentrum lima*, *Prorocentrum rhathymum, Prorocentrum emarginatum, Ostreopsis* cf. *ovata*, *Coolia* spp., *Amphidinium* spp. and *Gambierdiscus* spp. Seawater temperature at Arraial do Cabo varies from 14 to 27 °C due to coastal upwelling, while at Cairu, Bahia seawater temperature varies between 26 and 29 °C and at the latter location the species was found in tide pools.

4. Discussion

4.1. Taxonomy

The taxonomy of the genus *Prorocentrum* is mainly based on general cell shape, symmetry, presence or absence of spines, ornamentation and the pattern of pore distribution (Hoppenrath et al., 2013). Recently the morphology of the periflagellar area and its platelets is gaining more importance in the taxonomy of *Prorocentrum* species, besides the difficulties in observing them (Hoppenrath et al., 2013). When cells are elliptical and smooth without any obvious character, species identification is a challenging task, as these characters may also be considered variable within

a species (Nagahama et al., 2011; Hoppenrath et al., 2013; Zhang et al., 2015). The morphology of *P. caipirignum* is similar to *P. lima*

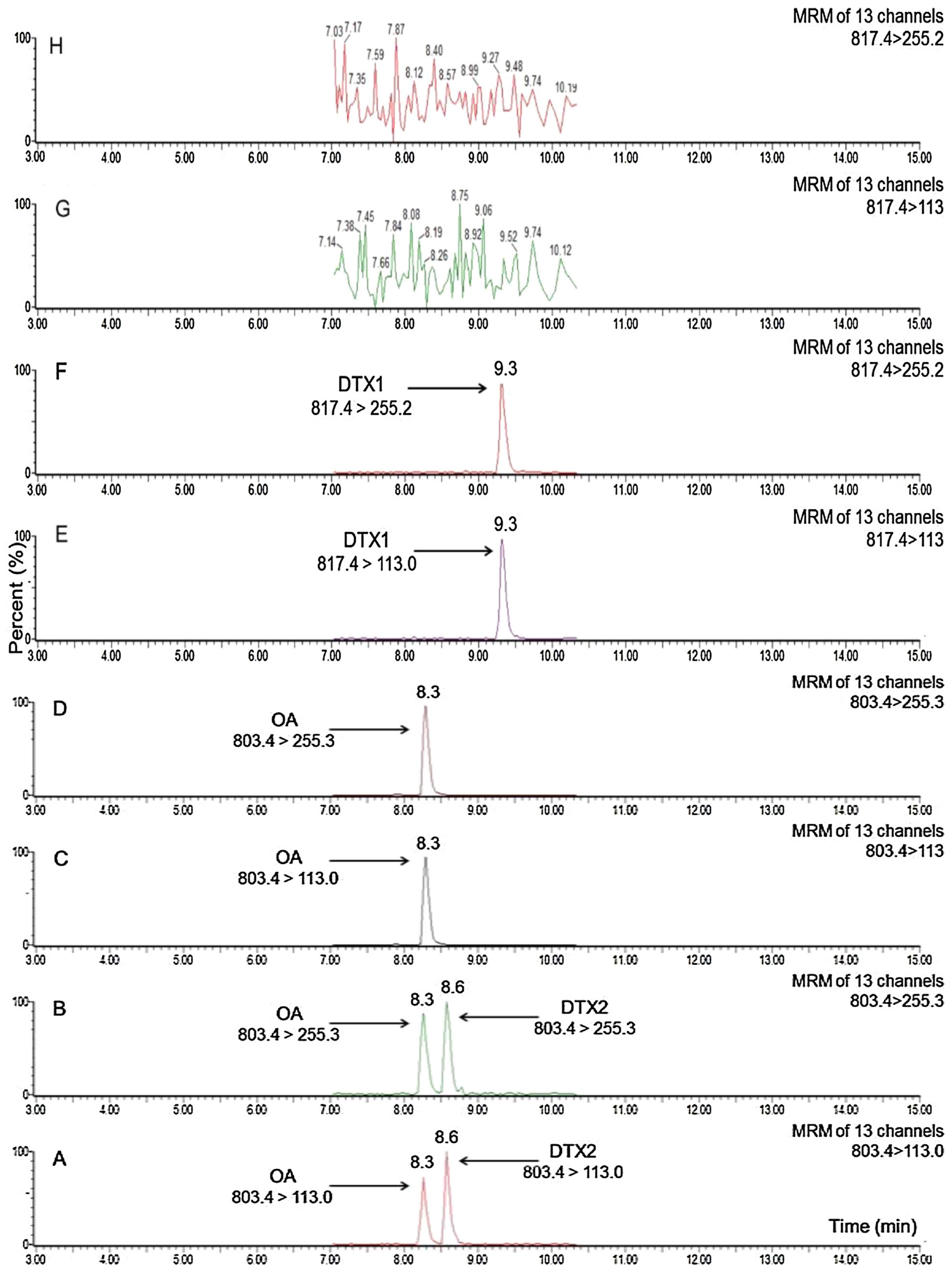


Fig. 8. Multiple reaction monitoring (MRM) chromatograms of: [A and B] okadaic acid (OA) and dinophysistoxins-2 (DTX2); DTX1 [E and F] reference standards, and of OA in *Prorocentrum caipirignum* strain LCA-B4 extract [C and D]. Neither DTX1 [G and H] and DTX2 [C and D] were detected.

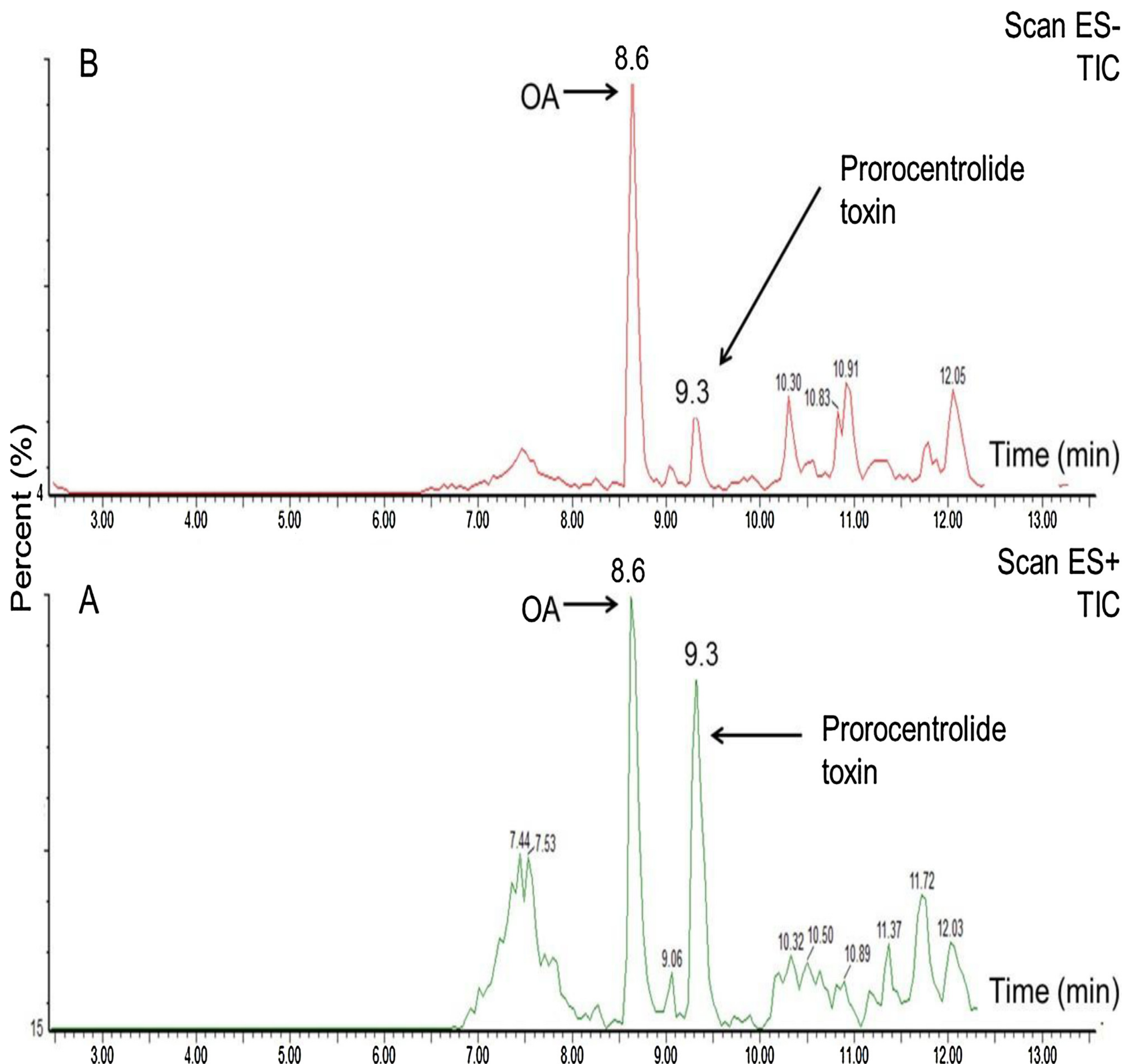


Fig. 9. Total ion count (TIC) chromatograms from (A) positive and (B) negative electrospray (ES) ionisation mode analyses of *Prorocentrum caipirignum* strain LCA-B4 extract.

but the two species can be differentiated by the general cell shape that is elliptical in *P. caipirignum* and hence being wider in the middle of the longitudinal axis, while according to the redescription of *P. lima* (Nagahama and Fukuyo, 2005), in that species it is tapering toward the anterior margin. The morphological differences between the two species were considered appreciable by Zhang et al. (2015) as to define different morphotypes of *P. lima*, which are considered as different species in the current study. Moreover, the width of the anterior region of the cell is broader in *P. caipirignum* than in *P. lima*. Cell shape is more symmetrical antero-posteriorly in *P. caipirignum*.

Cell shape was considered variable in *P. lima* as ellipsoid, ovoid or round and the species was described by Nagahama et al. (2011) as broad in the middle region, tapering toward the anterior margin, and round in the posterior margin. Ovoid is the main cell shape of *P. lima* (Hoppenrath et al., 2013), that is broader below the middle region of the cell, while only the round or broadly ovate form (that corresponds to *P. arenarium* Faust, 1994) is broad in the middle region of the cell. Aligizaki et al. (2009) refer to a "*P. lima* species complex" based on morphological variability, however, cells in Fig. 6A and B from Aligizaki et al. (2009) look more similar to *Prorocentrum hoffmannianum* in our opinion. Luo et al. (2017) also suggested that specimens in Fig. 6H from Aligizaki et al. (2009) might be *P. maculosum* instead. Therefore, although some morphological variability in *P. lima*, including variable cell shape and thecal pore shape is possible, this variability may not be as wide as previously perceived as more than one species were considered as *P. lima*.

Prorocentrum lima was described as *Cryptomonas lima* by Ehrenberg (1860) at the beginning of the time of taxonomy of dinoflagellates and not following the current criteria established by the nomenclature codes. Its basionym was redescribed (Nagahama and Fukuyo, 2005) based on Ehrenberg original drawings and wild and cultured cells from the type locality in the Bay of Naples, Italy. During all these years, the name *P. lima* was extensively used all over the world considering that this was a cosmopolitan species inhabiting both tropical and temperate areas. With the advent of molecular works, cases where a species

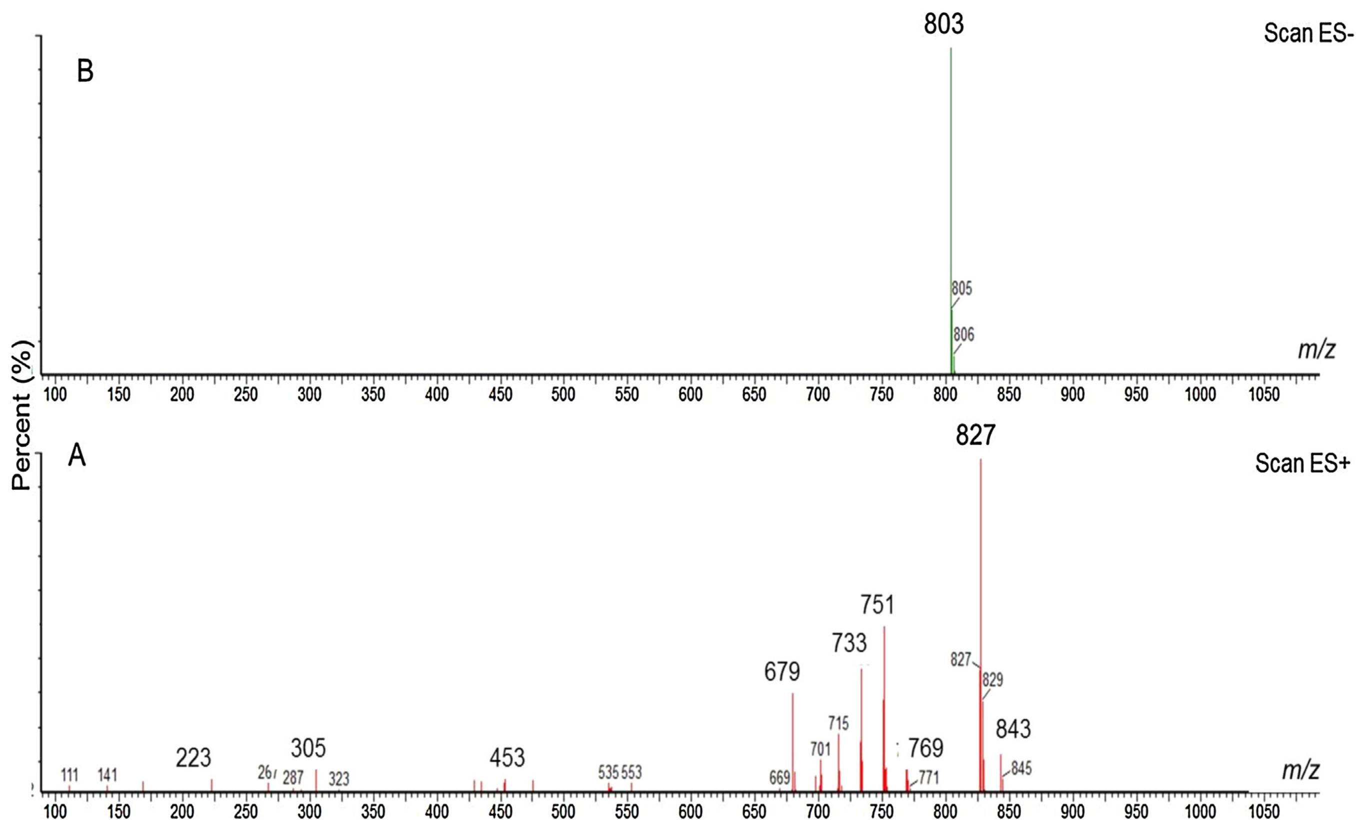


Fig. 10. Full scan, positive (A) and negative (B) electrospray (ES) ionisation mode mass spectra of okadaic acid (OA) eluting at 8.6 min as presented in Fig. 9. *Prorocentrum caipirignum* strain LCA-B4 extract.

previously considered cosmopolitan was split in more than one species became common in many protists. The planktic diatom *Skeletonema costatum* (Greville) Cleve, 1873 was considered for a long time a cosmopolitan species inhabiting both tropical and temperate seas, but after careful morphological studies combined with molecular work it was split into several new species (Medlin et al., 1991; Sarno et al., 2005; Zingone et al., 2005) and the diversity and biogeography of that genus was studied by Kooistra et al. (2008) showing that some species were found only in temperate waters while others in tropical or subtropical areas. A similar case was the dinoflagellate genus *Coolia* which was considered monospecific for many years and now comprises several species. Among them, *C. monotis* and *C. malayensis* are hardly distinguishable by morphology, but are easily differentiated genetically and the former has a temperate distribution while the later is more subtropical or tropical. Then, it is reasonable that more than one species may exist under the name *P .lima*.

Nagahama et al. (2011) studied cultured strains labeled as *P. lima* isolated from various places and although they considered it was one variable species, the presence of two or three species could be inferred from their data: One species from temperate waters, including the Mediterranean Sea where the type locality of *P. lima* is, that forms a monophyletic clade and has a smaller length-to width ratio than a second species including strains from tropical origin which are grouped in a different monophyletic clade with two branches. These two “species” correspond to respectively *P. lima* type II and *P. lima* type I according to Boopathi et al. (2015). If the conspecificity of these clades may be a reason for debate, it is easier to recognize that the name *P. lima* was applied to more than one species in the following cases: 1) *Prorocentrum arenarium* Faust, that was considered a synonym of *P. lima* (Nagahama et al., 2011). It was described based solely on morphology (Faust, 1994) and cells presenting the corresponding morphology (PAEU_01 and VGO776) appear in a third well defined clade distinct from the clade with strains from the type locality of *P. lima* and hence *P. arenarium* should be considered a good species and not a synonym of *P. lima*. 2) The name *P. lima* was also applied to *P. caipirignum* by Zhang et al. (2015). 3) When Nagahama and Fukuyo (2005) redescribed *C. lima*, they used samples from the type locality, but in their Fig. 1D they showed the left thecal plate of a strain from the Philippines which has a cell shape distinct from *P. lima* being more elliptical than ovoid, resembling *P. caipirignum*.

The periflagellar area of *P. lima* is hardly distinguished from that of *P. caipirignum*. Comparing the periflagellar area of *P. lima* from the type locality as described by Nagahama and Fukuyo (2005) with that of *P. caipirignum*, the main difference is the relative size of platelets 5 and 6 (g and e in Nagahama and Fukuyo, 2005). In *P. caipirignum* platelet 5 is bigger than 6, while the opposite occurs in *P. lima*. Another difference may be regarding platelet 8 (or c in Nagahama and Fukuyo, 2005), that is part of platelet 2 in *P. caipirignum* (Fig. 5), and not a separate platelet. However, this could be a problem of interpretation as other authors considered them as two different platelets, while in this work they appear as a single platelet. In Fig. 5 the periflagellar platelets are partially dissected and it can be seen that there is no suture between platelet 2 and what is described as platelet 8 in other species. Platelet 7 was not observed in *P. caipirignum*.

P. caipirignum is similar to *Prorocentrum vietnamensis* Yoo & Fukuyo (Yoo et al., 2004), from which it can be differentiated by the cell shape that is elliptical in *P. caipirignum* while *P. vietnamensis* is rod-shaped with parallel cell sides. *Prorocentrum vietnamensis* is 42 to 50 μm long and 36 to 42 μm wide, and hence considerably bigger than *P. caipirignum* from Brazil. The number and distribution of marginal and thecal pores are similar in both species. The shape of the periflagellar area of *P. vietnamensis* was described as wide V-shaped but that of *P. caipirignum* is more acute in a way that

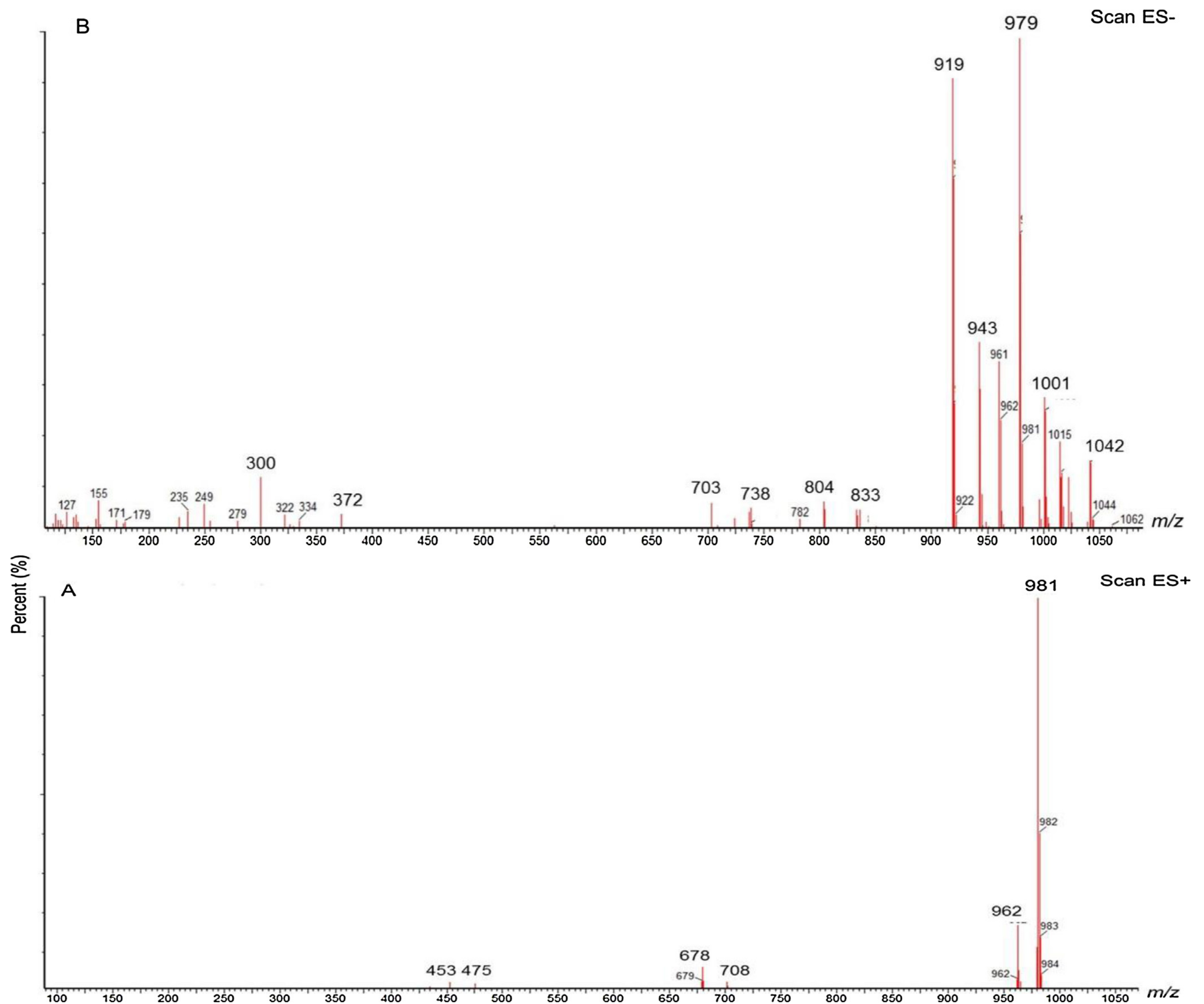


Fig. 11. Full scan, positive (A) and negative (B) electrospray (ES) ionisation mode mass spectra of the prorocentrolide toxin eluting at 9.3 min as presented in Fig. 9. *Prorocentrum caipirignum* strain LCA-B4 extract.

makes the one of *P. vietnamensis* more as U-shaped than V-shaped. The periflagellar platelets of that species were not described, hindering any comparison.

Prorocentrum caipirignum was observed and studied by several authors which considered it as different species. 1) Strain K-0625 from Malaysia was studied and identified by Mohammad-Noor et al. (2007) as *Prorocentrum arenarium* Faust and is now kept at the The Norwegian Culture Collection of Algae (NORCCA) where it is labeled as *P. lima* and *P. arenarium* as junior synonym, as this species was considered a synonym of *P. lima* by Nagahama et al. (2011). 2) Strain SE10 from Hainan Island, China was identified as *P. lima* morphotype 4 (Zhang et al., 2015) and then revised as *P.* cf. *maculosum* by Luo et al. (2017) together with strains SE10, TIO11, TIO138, TIO102 and TIO139 also from the same area in the South China Sea (Luo et al., 2017). 3) Strain PMHV-1 from Cuba was identified as *Prorocentrum maculosum* (Herrera-Sepúlveda, 2014; Herrera-Sepúlveda et al., 2015).

Besides those strains, Nguyen et al. (2004) showed SEM images of an elliptical *Prorocentrum* from Vietnam which is probably *P. caipirignum*. Unfortunately, only one single cell was observed and the information provided was not enough to get to a conclusion about its identity. These authors remark that the pores were kidney shaped as in *P. maculosum*. Morquecho-Escamilla et al. (2016) when described strain PMHV-1 from Cuba identified it as *P. maculosum* based on the kidney-shaped pores, although they reported that this was a variable character in their strain. The pore shape in these closely related *Prorocentrum* species (*P. lima*, *P. maculosum*, *P. caipirignum*) might be more variable than previously anticipated and this variability has probably contributed to the difficulties in identifying these morphologically similar species.

4.2. Phylogeny

The evolutionary divergence between sister species proceeds at variable rates depending on the studied organisms, and the factors that drive speciation are still poorly known (Logares et al., 2007). Ribosomal RNA genes array and intergenic regions (e.g. ITS) are widely used in taxonomic studies given that the phylogenetic relationships based on these markers agree to a large extent not only with traditional morphospecies but also differentiate cryptic/pseudocryptic ones (e.g. Vandersea et al., 2012; Hariganeya et al.,

Fig. 12. Selected ion recording (SIR) chromatograms of the prorocentrolide toxin from electrospray (ES) positive (m/z 980.5; [M+H]+) and negative (m/z 978.5; [M-H]-) ionisation mode analyses of a *Prorocentrum caipirignum* extract (mobile phase flow rate of 0.45 mL min^{-1}).

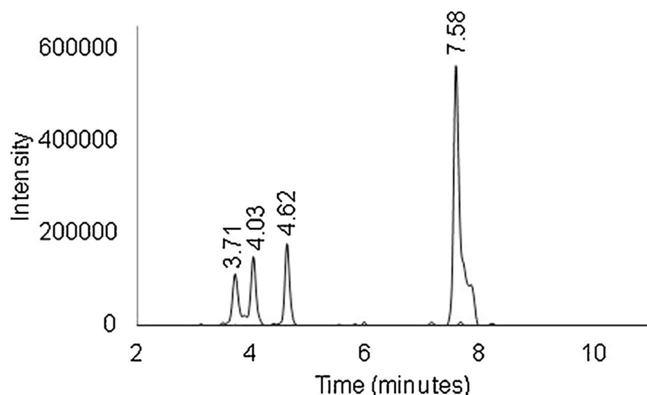


Fig. 13. LC-HRMS chromatograms from toxin standards OA, DTX1, DTX2 and PTX2 used in the analyses of *Prorocentrum caipirignum* strain UFBA064 extract.

2013). Nonetheless, in some cases, the actual reproductive isolation and morphological differences between closely related species are not mirrored by genetic results based on traditional ribosomal markers, which display resolution at different hierarchical levels (Edvardsen et al., 2003; Logares et al., 2007). In this sense, ribosomal RNA genes (LSU, SSU) are extensively used to assign putative microalgae species but ITS rDNA diverges faster during speciation, provides better resolution between recently diverged taxa, and it has been proposed as an appropriate locus for DNA barcoding of dinoflagellate species (Litaker et al., 2007).

In the present study, the *P. caipirignum* clade was supported by ITS phylogenetic analyses as a sister species of *P. hoffmannianum* species complex but LSU displayed much lower resolution between both clades. Regarding LSU rDNA sequences, the distance values between *P. caipirignum* and those of the closest species, the *P. hoffmannianum* species complex clade, were <2% in a fragment of 561 bp. This value would be in the same order as those observed within the *P. hoffmannianum* clade (0–1.86%; Herrera-Sepúlveda et al., 2015), among isolates of different geographic origins. These authors stated that based on morphological and genetic results, isolates belonging to *P. hoffmannianum* and *P. belizeanum* could be considered conspecific. Notwithstanding, the key issue is that within those *P. hoffmannianum*/*P. belizeanum* sequences genetic distances calculated based on the ITS rDNA fragment were below 2%, whereas significantly larger distances (~7–9%) were found between ITS sequences of *P. caipirignum* and the two closest clades (*P. hoffmannianum* and *P. lima* "morphotype 5" of Zhang et al., 2015).

These genetic differences using ITS rDNA support *P. caipirignum* as a new species and not just a genetically separated "*P. lima* morphotype". In the ITS phylogeny the Brazilian strains of *P. caipirignum* formed a clade with strains originally isolated from Cuba and Hainan Island, South China Sea, whereas in the LSU the strains were grouped with sequences from Cuba, Hainan Island and Malaysia. Therefore, it is likely that this new toxic species has a broad distribution including other tropical and subtropical regions worldwide.

Previous works on the relationships among *P. lima* isolates revealed a wide genetic variability that largely surpasses the proposed limit of 4% in the ITS rDNA region (0.04 substitutions per

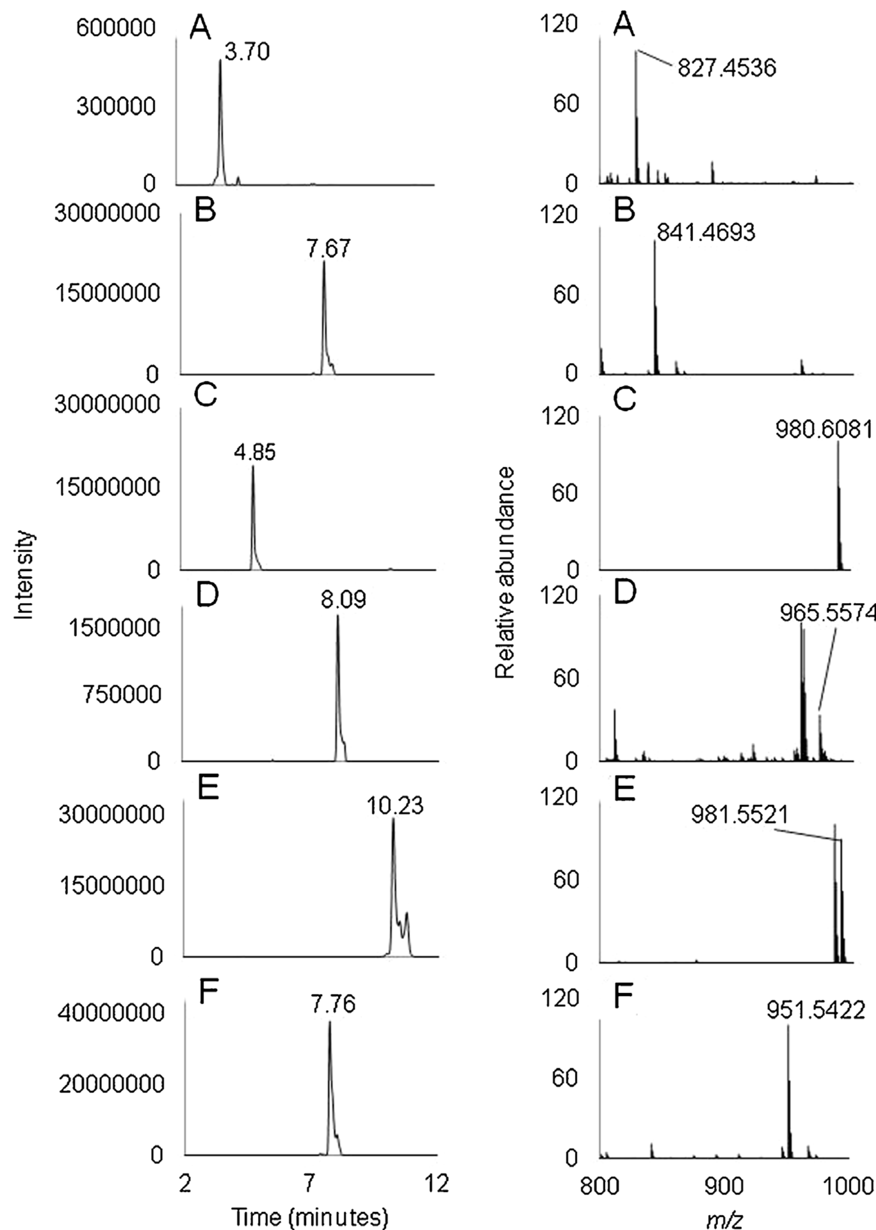


Fig. 14. LC-HRMS chromatograms (left) and mass spectrum (right) from *Prorocentrum caipirignum* strain UFBA064 corresponding to: (A) OA, (B) Methylokadaate, (C) Prorocentrolide, (D) compound 7/8*, (E) compound 9 and (F) compound 12/OA-D8-diol-ester*.
*Compounds 7 and 8 are analogs with identical molecular weight as well as compound 12 and OA-D8-diol-ester. Due to the lack of standards for these compounds it is not possible to determine which is the analogue present in the sample.

site of uncorrected *p*-distances) to recognize most free-living dinoflagellate species (Litaker et al., 2007). Herrera-Sepúlveda et al. (2015) reported that in *P. lima* the genetic distance between ITS sequences of strains from different regions (Pacific, Caribbean and Atlantic) covered a range of 0–12%. Five "*P. lima* morphotypes", not formally identified as separate species but only as a "*P. lima* complex" were described by Zhang et al. (2015). These authors examined the morphological and molecular characteristics of *P. lima* isolates from the South China Sea and revealed that these "*P. lima* morphotypes" were genetically separated. Our isolates of *P. caipirignum* clearly correspond with the strain identified by these authors as "*P. lima* morphotype 4" (SE10). Consistent morphological differences (e.g. in cell size and shape) were found between these morphotypes, but Zhang et al. (2015) considered they fell within the description of *P. lima*. Nevertheless, Luo et al. (2017) differentiated morphotypes 4 and 5 from *P. lima* sequences in their LSU and ITS phylogenies, and renamed them as *P.* cf. *maculosum* and *P. maculosum*, respectively. The same authors discussed the possibility that *P. maculosum* and *P. hoffmannianum* could be conspecific. Further identification of each separate clade was not intended.

Regarding the molecular characterization, Zhang et al. (2015) compared fragments of similar length to our study (580 nt in LSU and 521 nt in ITS) to determine the genetic distances between "*P. lima* morphotypes", and found a range of *p*-distances of 0.17% to 6.38% in LSU and 0.56% to 16.38% in ITS. The same authors prompted the need for an appropriate genetic value for species-level discrimination to clarify the relationships between "*P. lima* morphotypes". Nevertheless, considering that universal limits cannot be applied in every case, as each genus may display

different average genetic divergence and evolution rates, Zhang et al. (2015) referred to the "*P. lima* morphotypes" as a "*P. lima* complex".

When examining genetic distances between currently accepted *Prorocentrum* species it appears difficult to provide a *p*-distance value to differentiate among closely related species. To illustrate this we examined the examples of *P. minimum*/*P. dentatum* and *P. micans*/*P. rhathymum* based on full length ITS1-5.8S-ITS2 records in GenBank. Intraspecific genetic distances in *P. minimum* (n = 26) ranged from 0% to 6.6%, whereas their genetic distances in relation to *P. dentatum* (only two sequences) were 0.3-5.9%. Similar calculations for *P. micans* (n = 20) and *P. rhathymum* (n = 10) yielded intraspecific average genetic distances of 2.5% and 2.4% respectively, with an interespecific average distance of 9.9% (7.1-13.6%). Thus, even if these species pairs belong to the second major clade of the genus *Prorocentrum* and can have different evolutionary rates to those of *P. lima* and *P. hoffmannianum* complexes and *P. caipirignum*, their comparison and the results presented herein point out that different boundaries at intra- and interspecific levels are observed within the genus, but *p*-distances among *P. caipirignum* towards *P. lima* "morphotype 5" and *P. hoffmannianum* complex equate or even surpass the interespecific variations between *Prorocentrum* species.

4.3. Toxins

Both strains of *P. caipirignum* produced OA and no evidence of DTX1 and DTX2 was found. Other benthic *Prorocentrum* species that produce OA include *P. lima*, *P. hoffmannianum*, *P. concavum*, *P. faustiae*, *P. maculosum* and *P. rhathymum* (Hoppenrath et al., 2013). PTX2 presence was investigated in the UFBA strain and was not found. OA concentrations in each strain were quite different and while in strain UFBA064, 0.15 pg OA cell^{-1} were found, strain LCA-B4 produced 20 pg OA cell^{-1}. Both strains were analyzed at the exponential phase of growth. When cell pellets from strain LCA-B4 were boiled to inhibit the hydrolytic conversion of OA and DTX water soluble derivatives (e.g., DTX4s), 2 pg OA cell^{-1} were measured. The difference between the concentrations of free and total OA content in heat treated and non-boiled cells was most likely due to a biotransformation of sulphated OA derivatives and diol esters to the parent OA toxin (Quilliam et al., 1996), although we cannot ensure that the enzymatic hydrolysis of OA precursors to the respective parent toxins was complete. Light and temperature conditions used to grow both strains were similar and the difference observed in OA cell quotas might be caused by intraspecific variability.

Recently Luo et al. (2017) reported on toxin profiles of *Prorocentrum* species isolated from Hainan Island, South China Sea and the strains identified by these authors as *P.* cf. *maculosum* (TIO11, TIO138, TIO102, TIO139) corresponded to *P. caipirignum* and were shown to produce OA but no DTX1, as the Brazilian strains.

Methylokadaate, compounds 7/8, 9 and 12 (named OA-D8-diol-ester) were found in the UFBA064 strain. These compounds were not investigated in the strain LCA-B4. The presence of several OA diol esters has already been confirmed in other *Prorocentrum* species and the OA congeners numbered 5, 6, 7, 8, 9, 10 and 12 have been previously detected in *P. lima* and number 13 has been detected in *P.hoffmannianum* species complex (as *P. belizeanum*) (Paz et al., 2007). The macrocycle fast acting toxin prorocentrolide was found in both strains. It is an unusual nitrogen-containing macrolide originally identified in a *Prorocentrum lima* strain isolated from Japan by Torigoe et al. (1988). Prorocentrolide B was later identified in *P. maculosum* (but this identification may be doubtful and the species might be *P. hoffmannianum*, see Hoppenrath et al., 2013) isolated from the Caribbean (Hu et al., 1996). Prorocentrolides contain a cyclic imine moiety, like spiro-prorocentrimine, gymnodimine, spirolides pinnatoxins and pteriatoxins (Munday et al., 2012). The toxicological and pharmacological effects of these "fast-acting" toxins are not yet fully understood, though they are not phosphatase inhibitors, unlike their co-metabolites, the DSP toxins (Hu et al., 1996). Interestingly, two strains of *P. lima* isolated from eastern Canada and a *P. lima* strain isolated from Rio de Janeiro, Brazil (UNR-01) were shown to produce DSP toxins, but no prorocentrolide derivatives were observed in these strains (Hu et al., 1996; Nascimento and Morris, unpublished data). Further investigations regarding the production of prorocentrolide by strains from these closely related species of *Prorocentrum* (*P. lima*, *P. maculosum*, *P. caipirignum*, *P. hoffmannianum*) are necessary to verify the production of these compounds by different species.

5. Conclusions

Prorocentrum caipirignum is a new species closely related to *P. lima* species complex, which may represent more than one species. Based on morphology and phylogeny, the species may be a result of a recent process of sympatric speciation as it was found in distant areas coexisting with morphologically and molecularly distinguishable *P. lima*, like Brazil, Cuba, China and Indonesia. *Prorocentrum caipirignum* was identified by diverse authors as *P. maculosum*, *P. arenarium* or as a morphotype of *P. lima* (and later renamed as *P.* cf. *maculosum*). Although these are morphologically similar species (and *P. arenarium* was considered a synonym of *P. lima* by Nagahama et al., 2011), *P. caipirignum* and *P. lima* can be differentiated by morphology. The elliptical cell shape of *P. caipirignum* is a consistent character to distinguish this species from the ovoid *P. lima* and *P. maculosum*. ITS and LSU rDNA data support *P. caipirignum* as a new species. The Brazilian strains of *P. caipirignum* formed a clade with strains from Cuba, Hainan Island and Malaysia and it is therefore likely that this new species has a broad tropical distribution. *Prorocentrum caipirignum* is a toxic species that produces okadaic acid and the fast acting toxin prorocentrolide.

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References

Aligizaki, K., Nikolaidis, G., Katikou, P., Baxevanis, A.D., Abatzopoulos, T.J., 2009. Potentially toxic epiphytic *Prorocentrum* (Dinophyceae) species in Greek coastal waters. Harmful Algae 8 (2), 299–311.

Alves-de-Souza, C., Cornet, C., Nowaczyk, A., Gasparini, S., Skovgaard, A., Guillou, L., 2011. *Blastodinium* spp. infect copepods in the ultra-oligotrophic marine waters of the Mediterranean Sea. Biogeosciences 8 (8), 2125–2136.

Boopathi, T., Faria, D.G., Cheon, J.-Y., Youn, S.H., Ki, J.-S., 2015. Implications of high molecular divergence of nuclear rRNA and phylogenetic structure for the dinoflagellate *Prorocentrum* (Dinophyceae, Prorocentrales). J. Eukaryot. Microbiol. 62 (4), 519–531.

Bravo, I., Fernández, M.L., Ramilo, I., Martínez, A., 2001. Toxin composition of the toxic dinoflagellate *Prorocentrum lima* isolated from different locations along the Galician coast (NW Spain). Toxicon 39 (10), 1537–1545.

Chomczynski, P., Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat. Protoc. 1 (2), 581–585.

Edvardsen, B., Shalchian-Tabrizi, K., Jakobsen, K.S., Medlin, L., Dahl, E., Brubak, S., Paasche, E., 2003. Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analyses of rDNA. J. Phycol. 39, 395–408.

Ehrenberg, C., (1860). Nachtrag zu Hrn. Ehrenbergs Mittheilung vom 8. Decbr. 1859. Verzeichniss der Leuchtthierchen. Monutsberichte der Kb'niglichen Preitssischen Akademie der Wissenschaften zu Berlin 1859, 791–793.

Faust, M.A., 1994. Three new benthic species of *Prorocentrum* (Dinophyceae) from Carrie Bow Cay, Belize *P. sabulosum* sp. nov., *P. sculptile* sp. nov., and *P. arenarium* sp. nov. J. Phycol. 30, 755–763.

Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. J. Phycol. 21, 662–664.

Gerssen, A., Mulder, P.P.J., McElhinney, M.A., De Boer, J., 2009. Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. J. Chromatogr. A 1216, 1421–1430.

Glibert, P.M., Burkholder, J.M., Kana, T.M., 2012. Recent insights about relationships between nutrient availability, forms, and stoichiometry, and the distribution, ecophysiology, and food web effects of pelagic and benthic *Prorocentrum* species. Harmful Algae 14, 231–259.

Guillard, R.R.J., 1995. Culture methods. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. IOC Manual and Guides No 33. UNESCO, pp. 45–56.

Guiry, M.D., (2017). *Prorocentrum*, In: Guiry, M.D.G., G.M. (Ed.), Algaebase. Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=109566, 2016 ed. World-wide electronic publication, National University of Ireland, Galway.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hariganeya, N., Tanimoto, Y., Yamaguchi, H., Nishimura, T., Tawong, W., et al., 2013. Quantitative PCR method for enumeration of cells of cryptic species of the toxic marine dinoflagellate *Ostreopsis* spp. in coastal waters of Japan. PLoS One 8 (3), e57627. doi:http://dx.doi.org/10.1371/journal.pone.0057627.

Herrera-Sepúlveda, A., Medlin, L.K., Murugan, G., Sierra-Beltrán, A.P., Cruz-Villacorta, A.A., Hernández-Saavedra, N.Y., 2015. Are *Prorocentrum hoffmannianum* and *Prorocentrum belizeanum* (Dinophyceae, Prorocentrales), the same species? An integration of morphological and molecular data. J. Phycol. 51 (1), 1529–8817.

Herrera-Sepúlveda, A., 2014. Desarrollo y evaluación de métodos moleculares para la detección e identificación de dinoflagelados tóxicos y nocivos en las costas de México. Centro de Investigaciones Biológicas del Noroeste, S.C., La Paz, Baja California Sur, México (p. 120).

Hoppenrath, M., Chomérat, N., Horiguchi, T., Schweikert, M., Nagahama, Y., Murray, S., 2013. Taxonomy and phylogeny of the benthic *Prorocentrum* species (Dinophyceae) – A proposal and review. Harmful Algae 27, 1–28.

Hu, T.M., deFreitas, S.W., Curtis, J.M., Oshima, Y., Walter, J.A., Wright, J.L.C., 1996. Isolation and structure of prorocentrolide B, a fast-acting toxin from *Prorocentrum maculosum*. J. Nat. Prod. 59, 1010–1014.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17 (8), 754–755.

John, U., Litaker, R.W., Montresor, M., Murray, S., Brosnahan, M.L., Anderson, D.M., 2014. Formal revision of the *Alexandrium tamarense* species complex (Dinophyceae) taxonomy: the introduction of five species with emphasis on molecular-based (rDNA) classification. Protist 165 (6), 779–804.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. 23, 633–638.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16 (2), 111–120.

Kooistra, W.H.C.F., Sarno, D., Balzano, S., Gu, H., Andersen, R.A., Zingone, A., 2008. Global diversity and biogeography of *Skeletonema* species (Bacillariophyta). Protist 159, 177–193.

Kotob, S.I., McLaughlin, S.M., Van Berkum, P., Faisal, M., 1999. Discrimination between two *Perkinsus* spp. isolated from the softshell clam *Mya arenaria*, by sequence analysis of two internal transcribed spacer regions and the 5.8S ribosomal RNA. Parasitology 119, 363–368.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Lenaers, G., Maroteaux, L., Michot, B., Herzog, M., 1989. Dinoflagellates in evolution. a molecular phylogenetic analysis of large subunit ribosomal RNA. J. Mol. Evol 29 (1), 40–51.

Litaker, W.R., Vandersea, M.W., Kibler, S.R., Reece, K.S., Stokes, N.A., Lutzoni, F.M., Yonish, B.A., West, M.A., Black, M.N.D., Tester, P.A., 2007. Recognizing dinoflagellate species using its rDNA sequences. J. Phycol. 43 (2), 344–355.

Logares, R., Rengefors, K., Kremp, A., Shalchian-Tabrizi, K., Boltovskoy, A., Tengs, T., Shurtleff, A., Klaveness, D., 2007. Phenotypically different microalgal morphospecies with identical ribosomal DNA: a case of rapid adaptive evolution? Microb. Ecol. 53 (4), 549–561.

Luo, Z., Hua, Z., Krock, B., Lu, S., Yang, W., Gu, H., 2017. Morphology, molecular phylogeny and okadaic acid production of epibenthic *Prorocentrum* (Dinophyceae) species from the northern South China Sea. Algal Res.-Biomass Biofuels Bioprod. 22, 14–30.

Mayr, E., 1996. What is a species, and what is not? Philos. Sci. 63, 262–277.

Medlin, L.K., Elwood, H.J., Stickel, S., Sogin, M.L., 1991. Morphological and genetic variation within the diatom *Skeletonema costatum* (Bacillariophyta): evidence for a new species, *Skeletonema pseudocostatum*. J. Phycol. 27, 514–524.

Mohammad-Noor, N., Daugbjerg, N., Moestrup, O., Anton, A., 2007. Marine epibenthic dinoflagellates from Malaysia ? a study of five cultures and preserved samples based on light and scanning electron microscopy. Nordic J. Bot. 24 (6), 629–690.

Morquecho-Escamilla, L., Reyes-Salinas, A., Okolodkov, Y.B., 2016. Illustrated taxonomic guide of the marine dinoflagellate collection (CODIMAR)/Guía taxonómica ilustrada de a colección de dinoflagelados Marinas (CODIMAR). Centro De Investigaciones Biológicas Del Noroeste, S.C., La Paz, Baja California Sur, México.

Munday, R., Quilliam, M.A., LeBlanc, P., Lewis, N., Gallant, P., Sperker, S.A., Ewart, H.S., MacKinnon, S.L., 2012. Investigations into the toxicology of spirolides, a group of marine phycotoxins. Toxins 4, 1–14.

Murakami, Y., Oshima, Y., Yasumoto, T., 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. Bull. Japan. Soc. Sci. Fish. 48, 69–72.

Nagahama, Y., Fukuyo, Y., 2005. Redescription of *Crypotomonas lima*, collected from Sorrento, Italy, the basioniym fo Prorocentrum lima. Plankton Biol. Ecol. 52 (2), 107–109.

Nagahama, Y., Murray, S., Tomaru, A., Fukuyo, Y., 2011. Species boundaries in the toxic dinoflagellate *Prorocentrum lima* (Dinophyceae, Prorocentrales), based on morphological and phylogenetic characters. J. Phycol. 47 (1), 178–189.

Nascimento, S.M., Salgueiro, F., Menezes, M., Oliveira d, F.A., Magalhães, V.C.P., De Paula, J.C., Morris, S., 2016. *Prorocentrum lima* from the South Atlantic: morphological, molecular and toxicological characterization. Harmful Algae 57 (Part A), 39–48.

Nguyen, N.L., Larsen, J., Chu, V.T., 2004. Prorocentrales. In: Larsen, J., Nguyen, N.L. (Eds.), Potentially Toxic Microalgae of Vietnamese Waters. Council for Nordic Publications in Botany, Copenhagen, pp. 54–63.

Paz, B., Daranas, A.H., Cruz, P.G., Franco, J.M., Pizarro, G., Souto, M.L., Norte, M., Fernández, J.J., 2007. Characterisation of okadaic acid related toxins by liquid chromatography coupled with mass spectrometry. Toxicon 50, 225–235.

Percopo, I., Ruggiero, M.V., Balzano, S., Gourvil, P., Lundholm, N., Siano, R., Tammilehto, A., Vaulot, D., Sarno, D., 2016. *Pseudo-nitzschia arctica* sp nov., a new cold-water cryptic *Pseudo-nitzschia* species within the *P. pseudodelicatissima* complex. J. Phycol. 52 (2), 184–199.

Quilliam, M.A., Hardstaff, W.R., Ishida, N., McLachlan, J.L., Reeves, A.R., Ross, N.W., Windust, A.J., 1996. Production of diarrhetic shellfish poisoning (DSP) toxins by *Prorocentrum lima* in culture and development of analytical methods. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergovernmental Oceanographic Commission of UNESCO.

Richlen, M.L., Barber, P.H., 2005. A technique for the rapid extraction of microalgal DNA from single live and preserved cells. Mol. Ecol. Notes 5 (3), 688–691.

Sarno, D., Kooistra, W.H.C.F., Medlin, L.K., Percopo, I., Zingone, A., 2005. Diversity in the genus *Skeletonema* (Bacillariophyceae). II: An assessment of the taxonomy of S. costatum-like species with the description of four new species. J. Phycol. 41, 151–176.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10 (3), 512–526.

Torigoe, K., Murata, M., Yasumoto, T., Iwashita, T., 1988. Prorocentrolide, a toxic nitrogenous macrocycle from a marine dinoflagellate, *Prorocentrum lima*. J. Am. Chem. Soc. 110, 7876–7877.

Trainer, V.L., Bates, S.S., Lundholm, N., Thessen, A.E., Cochlan, W.P., Adams, N.G., Trick, C.G., 2012. *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. Harmful Algae 14, 271–300.

Vandersea, M.W., Kibler, S.R., Holland, W.C., Tester, P.A., Schultz, T.F., Faust, M.A., Holmes, M.J., Chinain, M., Litaker, R.W., 2012. Development of semi-quantitative PCR assays for the detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae). J. Phycol. 48, 902–915.

Yoo, J.S., Lee, J.H., Fukuyo, Y., 2004. *Prorocentrum vietnamensis* sp. nov. (Prorocentraceae Dinophyta): A new armored Dinoflagellate from Vietnam coastal waters. J. Plant Biol. 47 (2), 129–132.

Zhang, H., Li, Y., Cen, J., Wang, H., Cui, L., Dong, Y., Lu, S., 2015. Morphotypes of *Prorocentrum lima* (Dinophyceae) from Hainan Island, South China Sea: morphological and molecular characterization. Phycologia 54 (5), 503–516.

Zingone, A., Percopo, I., Sims, P.A., Sarno, D., 2005. Diversity in the genus *Skeletonema* (Bacillariophyceae): I. A reexamination of the type material of *S. costatum* with the description of *S. grevillei* sp. nov. J. Phycol. 41, 140–150.

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