The toxic benthic dinoflagellate *Prorocentrum maculosum* Faust is a synonym of *Prorocentrum hoffmannianum* Faust

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ABSTRACT

Three strains of the toxic benthic dinoflagellate *Prorocentrum hoffmannianum* were isolated in the Canary Islands (north-east Atlantic Ocean, Spain). The identity of the strains was determined by phylogenetic analyses of partial LSU rDNA (D1-D2 regions) but their morphology based on SEM images corresponded to *P. maculosum*. Their toxin profiles were analyzed by liquid chromatography and high resolution mass spectrometry analysis (LC–HRMS) on cell extracts and culture media. Okadaic acid and three analogs were detected in all strains. Rather, in culture media the detected compounds were variable among strains, two of them being okadaic acid analogs not found on cell extracts. As a result, the taxonomy of the species was revised and *P. maculosum* is proposed as a junior synonym of *P. hoffmannianum* whose description is emended.

Keywords

Benthic dinoflagellates, Canary Islands, dinophysistoxin, okadaic acid, *Prorocentrum*, LSUrRNA

1. Introduction

The species boundaries among the benthic species of the dinoflagellate genus *Prorocentrum*, *P. hoffmannianum* Faust (1990), *P. belizeanum* Faust (1993a), *P. maculosum* Faust (1993b), *P. sabulosum* Faust (1994) *P. tropicalis* Faust (1997) and *P. reticulatum* Faust (1997), have been considered problematic (Hoppenrath et al. 2013; Hoppenrath et al. 2014). All these species were formally described on external morphological characters, with the only mention to their photosynthetic nature, and the presence of a central pyrenoid and a posterior nucleus. Moreover, they were studied on wild specimens isolated from field samples in Twin Cays (Belize), and phenotypic plasticity in morphological characters used to delineate these species was often unknown. From previous molecular surveys in the genus *Prorocentrum* it has been found that strains from morphologically defined species like *P. belizeanum* and *P. hoffmannianum* show very close rDNA sequences (e.g. 98.9% similarity based on an alignment of 560 bp of the LSUrDNA; Murray et al., 2009). Recently, based on morphological and molecular data, Herrera-Sepúlveda et al. (2015) concluded that *P. hoffmannianum* and *P. belizeanum* might be considered conspecific. Although, as they formed slightly different subclades generally corresponding with their geographical origin these authors considered them to be a species complex. In this paper, *P. maculosum* was included to this group of species and it was proposed to be a synonym of *P. hoffmannianum*, the latter name having priority according to the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code) (McNeill et al., 2012).

Dinoflagellates of the genera *Prorocentrum* and *Dinophysis* are known producers of DSP (Diarrhetic Shellfish Poisoning) toxins, such as okadaic acid (OA) and dinophysistoxins (DTXs) (Lee et al., 1989; Yasumoto et al., 1989; Hu et al., 1992; James

et al., 1997; Daranas et al., 2001). The incidences of DSP syndrome appear to be rising, although this may be partly due to increasing knowledge about the disease and better surveillance programs (FAO, 2004). In addition, it must be noted that the existence of toxin-producing algae and toxic molluscs are frequently reported from new areas (Aune and Yndestad, 1993).

A number of naturally occurring derivatives of OA and DTXs have been reported in microalgae (Domínguez et al., 2010), and a large number of esters of OA have been described called 'OA diol esters', where the carboxylic group of OA is esterified with 6- to 10-carbons diol fragments. These compounds were found in dinoflagellates of *Prorocentrum* and *Dinophysis* genera (Yasumoto et al., 1989; Hu et al., 1992; Suárez-Gómez et al., 2001, 2005; Fernández et al., 2003; Suzuki et al., 2004; Miles et al., 2006; Torgersen et al., 2008; Paz et al., 2007a). In addition, *Prorocentrum* spp. may produce water-soluble derivatives of OA and DTX1 diol esters such as dinophysistoxin-4 (DTX4), dinophysistoxin-5 (DTX5a, b and c), where the diol esters are further conjugated with a polar side chain (Hu et al., 1995a,b; Quilliam and Ross, 1996; Cruz et al., 2006; Paz et al. 2007b and Vilches et al. 2012). *In vivo* pharmacological experiments showed that OA esters are as active as the free acid, although the latency period is significantly higher for the esters (Trujillo et al., 2001 and Fernández et al., 2002).

Herein, the presence of OA was confirmed in three *Prorocentrum hoffmannianum* strains isolated in the Canary Islands, by HPLC-MS analyses. This is the first study on the morphological and molecular taxonomy and toxin profiles of *P. hoffmannianum* strains in the area, with the aim of contributing to a better knowledge about the global distribution of *Prorocentrum* spp.

2. Materials and Methods

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91 *2.1. Source of specimens and culture conditions*

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93 Samples of diverse macroalgae were collected in a tidal pond at La Puntilla (28º
94 8.9' N, 15º 26'W), in Las Palmas, Canary Islands, in February 2010 (Fig. 1). Samples
95 were placed in plastic bottles with local seawater and shaken. Afterwards, the gross
96 materials were removed through a sieve and the remaining seawater transported to the
97 IEO laboratory in Vigo for cell isolation. Single cells were picked using a capillary
98 pipette with the aid of a Zeiss Invertoscop D microscope (Carl Zeiss AG, Germany) and
99 incubated in 96 microwell plates in K/2 medium (Keller et al. 1987) made with seawater
100 from Ría de Vigo, salinity adjusted to 34. Cultures were grown at 24 ºC, under a photon
101 irradiance of about 90 µmol quanta m^{-2} s^{-1} measured with a QSL-100 irradiameter
102 (Biospherical Instruments Inc. San Diego, CA, USA), with a 14:10 L:D photoperiod.
103 Three strains of *P. hoffmannianum* were established and labeled VGO1029, VGO1030
104 and VGO1031. The three strains were deposited at the Culture Collection of Microalgae
105 of the Instituto Español de Oceanografía in Vigo (CCVIEO).

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107 *2.2. Light microscopy*

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109 Cultured cells were observed under a Leica DMLA light microscope (Leica
110 Microsystems GmbH, Wetzlar, Germany) with differential interference contrast.

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112 *2.3. Sample preparations for SEM*

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Five mL of exponentially growing cultures were fixed with a solution of GA made in seawater to get a final concentration of 2%. After two hours at room temperature, they were rinsed three times with distilled water and dehydrated in a series of 30, 50, 75, 95 and 100% EtOH followed by 100% Hexamethyldisilazane. After being air dried overnight, they were coated with gold with a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a FEI Quanta 200 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

2.4. Molecular analyses: PCR amplification and DNA sequencing

Aliquots of cultures of three *P. hoffmannianum* strains (VGO1029, VGO1030, VGO1031) from La Puntilla and one of *P. elegans* isolated from La Palma Island (F. Rodríguez, 2012) were poured on a glass slide, and cells (2-5 individuals in each sample) were picked up with a micropipette, washed three times in distilled water and transferred to 200 μL tubes for immediate PCR amplification. The D1-D2 regions of the LSUrRNA gene were amplified using the pairs of primers D1R/D2C (5'-ACCCGCTGAATTTAAGCATA-3'/5'-ACGAACGATTTGCACGTCAG-3'; Lenaers et al. 1989). The amplification reaction mixtures (25 mL) contained 4 mM $MgCl_2$, 0.5 pmol of each primer, 0.8 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, California, USA) and approximately 2 μL of distilled water with *Prorocentrum* cells. DNA was amplified in an Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA) following the conditions in Lenaers et al. (1989). A 10 μL aliquot of each PCR reaction was checked by agarose gel electrophoresis (1% TAE, 50 V) and GelRed™ nucleic acid gel staining (Biotium, Hayward, CA, USA). 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 Centro de Apoyo Científico Tecnolóxico á Investigación (C.A.C.T.I., Universidade de Vigo, Spain) sequencing facilities. The D1-D2 sequences obtained in this study were deposited in GenBank (for Acc.Nos. see Fig. 3).

2.5. Phylogenetic analyses

LSU sequences were inspected and aligned using MUSCLE alignment in Geneious® Pro 5.6.6 (Biomatters Ltd.). D1-D2 alignments included 538 positions. *Adenoides eludens* and *Amphidinium crassum* sequences were used to root the tree. The phylogenetic relationships were determined using Bayesian Inference (BI) and Maximum Likelihood (ML) methods. Phylogenetic model selection (ML) was performed on MEGA 7 software. A K2+G model (Kimura, 1980) was selected with gamma shape parameter = 0.57. BI analyses were performed with Mr. Bayes v3.2.4 (Huelsenbeck and Ronquist 2001), and in this case 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 number of generations used in these analyses was 1,000.000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization ("burn-in" phase). All final split frequencies were < 0.02. The phylogenetic tree was represented using the BI method, with posterior probabilities and bootstrap values from BI and ML, respectively.

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165 *2.6. Culture of Prorocentrum strains for toxin analysis*

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167 The three *P. hoffmannianum* strains were grown in 5 L flasks containing 2.5 L of
168 K medium (Keller et al., 1987) at 23 ºC, 35 salinity, and an irradiance of 60 µmol quanta
169 m^{-2} s^{-1} under a 18:6 L:D photoperiod. Cultures were incubated statically for 6 weeks. 2.5
170 L of each culture with a cell density average of 20,000 cells mL^{-1} were harvested by
171 filtration in a vacuum system through 1.6 µm glass fiber filters (GFF). Filters were
172 washed (2 x 15 mL) with sterile seawater and then stored at 4ºC. Collected cells were
173 used to estimate the toxin profile of each strain.

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175 *2.7. Toxin extraction*

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177 Cells were extracted with methanol in a sonication bath (Ultrasounds-HD, JP
178 Selecta, Spain) during 10 minutes with a power of 180 W (2 x 400 mL). Afterwards, the
179 solvent was evaporated in vacuum yielding a dark-green residue which was resuspended
180 in 10 mL of methanol and centrifugated during 15 minutes at 1500 rpm (Centrosix, JP
181 Selecta, Spain) in order to remove cell debris. On the other hand, to obtain the toxins
182 from culture media extracts (5 L) a Diaion HP20 resin column (4 x 30 cm) was used
183 following MacKenzie et al (2004). This resin is a non-polar copolymer styrene-
184 divynilbenzene adsorbent used in reverse phase chromatography. Once all the culture
185 media were passed through the resin, the organic compounds were desorbed with
186 methanol that was subsequently evaporated at low pressure yielding a yellow extract. The
187 toxin profiles of the six samples were analyzed to determine presence of OA and OA
188 derivatives included OA-diol-esters. Thus, the toxinological profile was carried out using

OA and OA diol-ether standards that were previously purified from cells of either *P. lima* (PL2V) or *P. belizeanum* (PBMA01) cultures, and subsequently fully identified by NMR spectroscopy and LC-MS/MS (Paz et al., 2007a).

2.8. Chemicals and materials

Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). Organic solvents used were ammonia solution 25% eluent additive for LC-MS (Scharlab S.L., Spain) and acetonitrile LC-MS grade (PanReac, AppliChem GmbH, Darmstadt, Germany)

A standard mix solution containing OA, DTX2, DTX1 and PTX2 (0.71, 0.40, 0.40 and 0.42 $ng \cdot \mu L^{-1}$ respectively) was prepared with individual standards from CIFGA Laboratories S.A. (Spain). A solution containing a mixture of OA congeners: OA, DTX-1, methyl okadaate, norokadanone, 2'-hydroxymethyl-allyl okadaate, 5'-hydroxy-2'-methylene-pent-3'-enyl okadaate, 7'-hydroxy-2',4'-dimethyl-hepta-2',4'-dienyl okadaate, 7'-hydroxy-4'-methyl-2'-methy- lene-hept-4'-enyl okadaate, 5',7'-dihydroxy-2',4'-dimethylene-heptyl okadaate, 5'-hydroperoxy-7'-hydroxy-2',4'-dimethylene-heptyl okadaate, 4'-formyl-2'-methylene-pent-4'-enyl okadaate (DTX-6), 7'-hydroxy-2'-methyl-hepta-2',4'-dienyl okadaate, prepared at IUBO Laboratory was used for qualitative identification of OA related metabolites (Paz et al., 2007a).

2.9. Toxin analyses

Liquid chromatography and high resolution mass spectrometry analysis (LC–HRMS) were carried out on a Thermo Scientific Dionex High-Speed LC coupled to

HRMS using an Orbitrap-Exactive mass spectrometer equipped with a HESI-II probe for electrospray ionization. Autosampler was kept at 15ºC. A X Bridge BEH C18 2.5 μm (2.1 mm x 50 mm) column was used for the separation of toxins at a flow rate of 400 μL min^{-1} and 40ºC. Mobile phase A was 0.05 v/v % ammonia in water (pH 11) and B was 0.05 v/v % ammonia in 90% acetonitrile. The gradient started at 25% of B, this percentage maintained for 1.5 min. Then it increased to 95% of B in 6 min and was kept 2 min at 95% B. Afterwards, % of B decreased to initial conditions (25%) in 3 min and it was maintained for 1.5 min to equilibrate the column. The total run time was 14 min.

The instrument was daily calibrated and operated in both positive and negative modes with and without all ion fragmentation (AIF) (NCE 65 eV). OA, DTX1 and DTX2 were analyzed in ESI negative mode. Analyses of the other OA analogs and OA-esters were performed in ESI positive mode. The mass range was *m/z* 100-1200 in full scan and AIF mode. The resolution in full scan and also in AIF mode was 140000 with a maximum injection time of 200 ms. Optimized parameters in positive and negative modes were spray voltage 3.250 KV, Capillary temperature 375ºC, sheath gas 45 (arbitrary units), auxiliary gas 15 (arbitrary units) and S-Lens RF level 55. The data were processed with Xcalibur 2.1 software (ThermoFisher Scientific, Bremen, Germany). To identify each toxin, the retention times of the analytes were compared with those of available reference standards (SD<3%). Peaks identity were confirmed by the exact diagnostic mass, the isotopic pattern and the mass accuracy (±5 ppm extraction window). Limit of detection (LOD) was defined as the amount of the analyte producing a signal-to-noise ratio (S/N) of 3. LOD value for OA with the instrumentation and analytical conditions applied during the present work was 6 pg.

3. Results

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240 *3.1. Morphology*

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242 The cells were ovoid in valve view with the maximum width behind the middle
243 region. The cells were 45-53 µm long (average 48, n=25) and 40-50 µm wide (average
244 43, n=25). The L/W ratio ranged from 1.01 to 1.18 (average 1.11). The valve surface
245 ornamentation was variable, usually from reticulate-foveate near the margins to smooth
246 in the middle (Fig. 2 A-B). It had scattered kidney-shaped pores of about 0.70 x 0.25 µm
247 that in many cases were pairs of two pores inside a curved depression (Fig. 2 C). It had
248 marginal pores in pairs oriented parallel to the edge of the valves (Fig. 2A-C). The centre
249 of the valve, in addition to be smoother than the periphery, was devoid of pores. The
250 intercalary band was smooth. The periflagellar area was wide V-shaped with collar of
251 variable size and platelet lists adjacent to the flagellar pores. It sometimes had a small
252 ridge at the edge of the periflagellar area making the area slightly asymmetric. Platelet 1
253 was triangular and had several deep depressions (Fig. 2 D). In old cells the intercalary
254 band and the collar were very pronounced (Fig. 2 E-F). Cells had two overlapped central
255 pyrenoids and a posterior nucleus (Fig. 2 G).

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257 *3.2. Molecular Phylogeny*

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259 *Prorocentrum* strains VGO1029, VGO1030 and VGO1031, showed identical
260 sequences for the D1-D2 region of LSU rRNA gene. The phylogenetic results (Fig. 3)
261 grouped the three strains in a clade with several *P. hoffmannianum* and *P. belizeanum*
262 sequences, with *P. caipirignum* (Nascimento et al. 2017) and *P.* cf. *lima* (morphotype 5;
263 Zhang et al. 2015) as sister groups. The three *Prorocentrum* strains from Canary Islands

matched exactly four *P. hoffmannianum* sequences in the alignment. The only record from GenBank labeled as *P. maculosum* showed 7 different bases (4 transitions and 3 transversions). This sequence was closest to a *P. lima* strain (PL1-11; DQ336188) that differed in 10 bases from *P. hoffmannianum* strains in the present study.

3.3. DSP profiles

Analyses of extracts from *P. hoffmannianum* (VGO1029, VGO1030 and VGO1031) cells confirmed the presence of okadaic acid, OA (**1**) *m/z* 827.4535 $[M+Na]^+$, a deoxyderivative of OA (**2**) *m/z* 811.4611 $[M+Na]^+$, 7'-hydroxy-2'-methyl-hepta-2',4'-dienyl okadaate (**3**) *m/z* 951.5446 $[M+Na]^+$, and 4'-formyl-2'-methylene-pent-4'-enyl okadaate (DTX-6) (**4**) *m/z* 935.5141 $[M+Na]^+$. In addition, analysis of media extracts showed the presence in the three studied strains of OA (**1**) and the diol esters 7'-hydroxy-2'-methyl-hepta-2',4'-dienyl okadaate (**3**) and 7'-hydroxy-2',4'-dimethyl-hepta-2',4'-dienyl okadaate *m/z* 965.5599 $[M+Na]^+$ (**5**); while 7'-hydroxy-4'-methyl-2'-methy- lene-hept-4'-enyl okadaate (**6)** *m/z* 965.5599 $[M+Na]^+$ was only detected in VGO1029 and VGO1030; and the OA deoxyderivative (**2**) was only detected in VGO 1030 (Fig. 4, Table 1).

4. Discussion

4.1. Morphology

Based on morphology, the three *Prorocentrum* strains from Canary Islands (VGO1029, 1030 and 1031) could be identified as *P. maculosum* Faust (1993), although the cells were slightly bigger than those of the original description. Faust (1993) reported

the pores as kidney-shaped as shown in her figure 3 that does not allow seeing more details than the outline. Zhou and Fritz (1993) in cultures of a strain previously identified as *P. concavum* by other authors, but identified by Maria Faust as *P. maculosum*, observed pores in doublets as easily observed in their figure 4 despite the low magnification. In the present study, the kidney-shaped pores in the three *Prorocentrum* strains were the result of these doublets that may appear as a single pore in a kidney-shaped depression. These doublets of pores, or kidney-shaped pores, have been observed also in other *Prorocentrum* species like *P. reticulatum* (Faust, 1997) where they were reported as kidney-shaped openings, and as oblong-shaped trichocyst pores in *P. sabulosum* (Faust, 1994). *Prorocentrum hoffmannianum*, *P. belizeanum*, *P. maculosum*, *P. sabulosum* , *P. tropicalis* and *P. reticulatum* are all very similar species sharing the type locality, Belize. The main difference among them is the degree of ornamentation, from the smoother *P. maculosum* to the most ornamented *P. reticulatum,* and the number of valve depressions (areolae). Although, the degree of ornamentation, size and shape are known to be very variable in *Prorocentrum* (Hoppenrath et al., 2013; Nagahama et al., 2011; Cohen-Fernández et al., 2006).

4.2. Molecular phylogeny

Molecular phylogeny points out to the monophyletic nature of the genus *Prorocentrum,* but with low statistical support (e.g. Tillmann et al., 2012). Several major clades have been identified in this genus with moderate/high support values (Hoppenrath et al., 2013; David et al., 2014). One of these major clades includes a number of species like *Prorocentrum belizeanum, P. bimaculatum, P. concavum, P. consutum, P. foraminosum, P. hoffmannianum* and *P. lima* (Hoppenrath et al., 2013), also with high probability support in this study.

Within this major clade, it is already evident that several sequences labeled as *P. belizeanum* and *P. hoffmannianum*, and the *P. maculosum* strains from the Canary Islands cannot be distinguished by LSU rDNA analyses and belong to a separate clade to other sister groups such as *P.* cf. *lima* (morphotype 5; Zhang et al. 2015), *P. caipirignum*, *P. arenarium* and *P. lima*. In this phylogeny there was also found a *P. lima* strain (PL1-11) which appeared to be basal to the *P. hoffmannianum/P. belizeanum* clade (as observed in previous studies; Hoppenrath et al., 2013), but closely related (a single transition base change) to a sequence identified as *P. maculosum* PMHV-1 (JQ638940). Nevertheless, to the best of our knowledge, the morphology of this strain has not been published yet and no morphological data can be checked with the Canary Islands strains of *P. maculosum* that matched the original description of this species (Faust 1993b). Herrera-Sepúlveda et al. (2015) in a study with different strains identified as *P. hoffmannianum* and *P. belizeanum* from different parts of the world, concluded that based on molecular sequence data (SSU, ITS, LSU and *cob*) and the subtle morphological differences, those species might be considered conspecific. Nevertheless, they found three different clades that corresponded to their geographical distribution (Belize, Florida-Cuba and Indian Ocean) but not to the two species and should be considered as a species complex. These results included *P. maculosum* from the Canary Islands in the *P. hoffmannianum* "species complex", in the so-called Florida-Cuba clade A (Herrera-Sepúlveda et al. 2015). After this result, it is suggested in the present study that the geographical labels of these molecular clades (or ribotypes) are actually not informative. At present, the overlapping of morphological traits and molecular data gathered from *P. belizeanum*, *P. hofmanniannum* and *P. maculosum* suggests that they are conspecific and should be designated as *P. hoffmannianum* as it has priority over the other species names.

Therefore, as *P. hoffmannianum* description has to include now morphological characteristics originally applied to other species, this species has to be emended.

4.3. Toxins

From a biochemical point of view, measured by its capability to produce different secondary metabolites, *Prorocentrum hoffmannianum* is clearly one of the most prolific dinoflagellate species ever studied. Metabolic profiling of the extracts obtained from the three different strains of *P. hoffmannianum* studied (VGO1029, VGO1030 and VGO1031) showed their ability to produce OA (**1**) as the most abundant toxin in all of them, as it is the case for other species of *Prorocentrum*. On the other hand, the deoxyderivative of OA (**2**) found here has not been previously detected in *P. lima* strains isolated in Galician coastal waters. Moreover, although (**2**) was detected in cell extracts of the three strains, it was only found in the media extracts of VGO1030. Regarding the ester derivatives of OA, compound (**3**) was found in both media and cell extracts of all strains, while (**4**) was only detected in cell extracts. On the contrary, esters (**5**) and (**6**) were unexpectedly only detected in media extracts of all strains with the exception of (**6**) in VGO1031. These results suggest that although the three strains shared a general toxin profile with other species of the *P. hoffmannianum* "species complex", their characterization using morphological traits and molecular data could be complemented with detailed toxin profiles from cell and media extracts to gain further information.

Thus, in addition to its well-known capability to biosynthesize the main DSP toxin, okadaic acid, several derivatives such as corozalic (Napolitano et al. 2010), belizeanic (Cruz et al., 2008) and 19-epi-okadaic (Cruz et al., 2007) acids have been isolated from this dinoflagellate although it has to be noted that it was originally classified as *P. belizeanum*. The same misperception is very likely for the study of other dinoflagellates

where okadaic acid was isolated (Morton et al., 1998). Also some of the most complex and bioactive macrolides known such as hoffmanniolide (Hu et al., 1999), belizeanolide (Napolitano et al., 2009) and belizentrin (Domínguez et al., 2014) have been isolated from this species. Moreover, large polyoxygenated polyketides such as belizeanic acid (Napolitano et al., 2009), included within the super-carbon-chain group of compounds have been discovered from the dinoflagellate described in this study.

Taxonomic appendix

Prorocentrum hoffmannianum Faust 1990 emend. S. Fraga

Cell shape is ovoid in valve view with the maximum width behind the middle region; cells are narrow at the anterior end. Cells are 40-60 µm long and 30-55 wide. Valve surface ornamentation is variable from foveate only near the valve margins, with the center of the valve smooth, to reticulate-foveate the whole valve. Valve pores have about 0.2 µm diameter and appear in doublets that can be fused forming kidney-shaped pores of 0.25 x 0.70 µm. The plate centre is devoid of pores. They are at the bottom of the depressions but, when the theca is foveate only near the margins, pores can be on the smooth part without depressions. When they are deeply foveate-reticulate it is difficult to observe the pores. It has marginal pores that, when the doublets can be observed, are placed parallel to the margin and they are present in all the marginal depressions. The periflagellar area is wide V-shaped. Six platelets were identified. Platelets 7 and 8 were not detected. Platelet 1 has a more or less triangular shape and has three or more very deep depressions. Other platelets are smoother.

Holotype: Figure 13 in Faust 1990.

Isotypes: Figure 14 in Faust 1990; Figures 1 -2 in Faust 1993a; Figures 1-2 in Faust 1993b; Fig. 2A of strain VGO1031.

Synonyms. *Prorocentrum belizeanum* Faust (1993a); *Prorocentrum maculosum* Faust (1993b).

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FIGURE LEGENDS

Figure 1. (A) Map of the East Atlantic archipelagos. (B) Map of the Canary Islands showing the locality where *Prorocentrum hoffmannianum* was found (arrow).

Figure 2. Scanning electron microscopy (A-F) of *Prorocentrum hoffmannianum* (strain VGO1031). (A) Right thecal view. (B) Left thecal view. (C) Details of thecal pores in doublets. (D) Periflagelar area. Plates 1 to 6, Accesory pore (ap), flagellar pore (fp), collar (arrow) and small ridge (asterisk). (E, F) Left thecal views of cells of different age showing variable size of intercalary bands and collars. (G) Light microscopy of *Prorocentrum hoffmannianum* (strain VGO1029). Py: pyrenoid; Nu: nucleus; Chl: chloroplast; Pu: pusule. Scale bars: A, B, E, F and G, 10μm; C and D, 5μm.

Figure 3. Phylogenetic tree obtained by BI model based on LSU rRNA sequences (D1-D2 region). The sequences derived from this study are highlighted in bold. Numbers at the branches are posterior probability and bootstrap percentages (n=1000) after BI and ML analyses, respectively. Support levels lower than .60/60% are denoted by hyphens.

Figure 4. Chemical structures of okadaic acid related metabolites.

Figure
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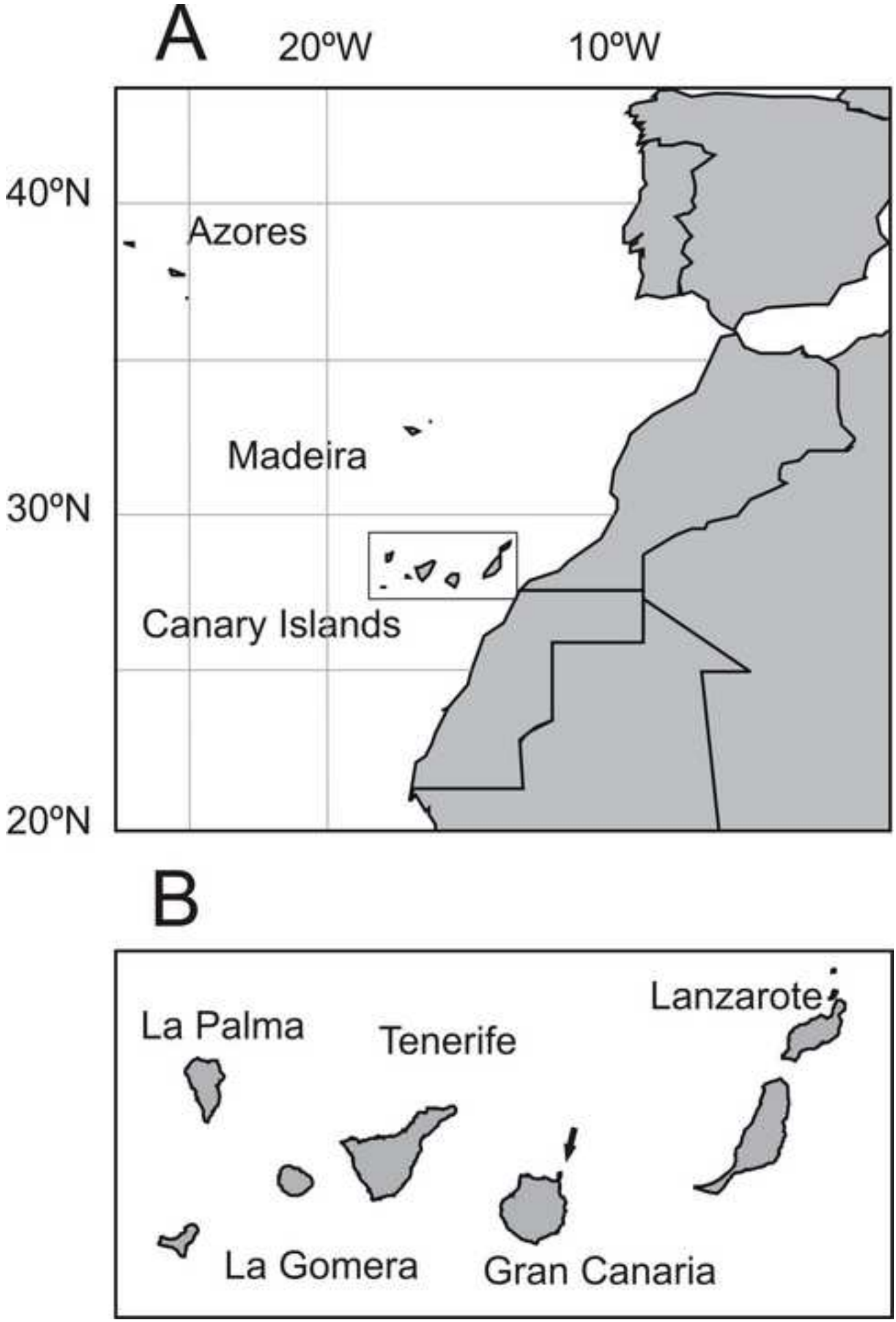

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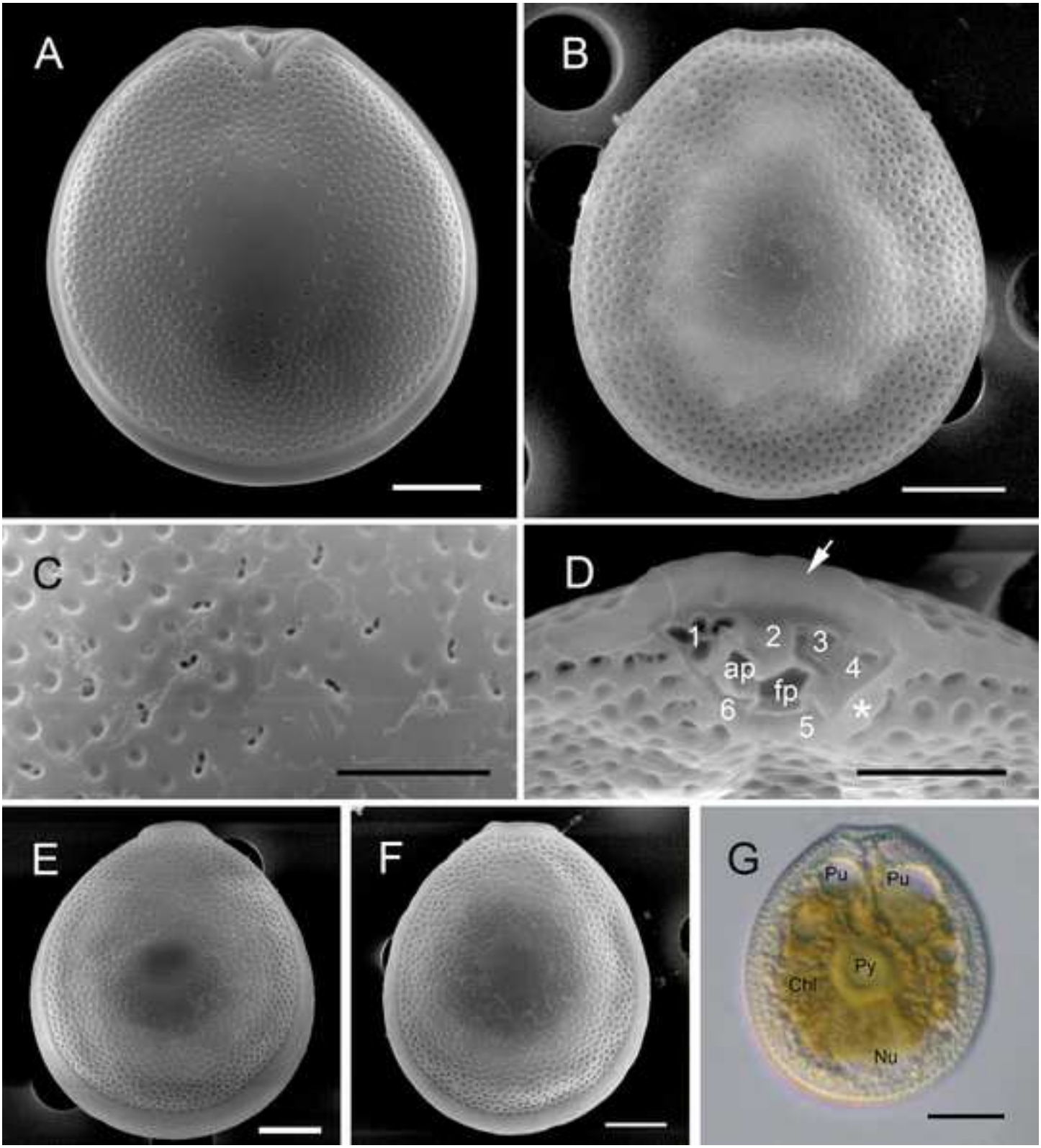

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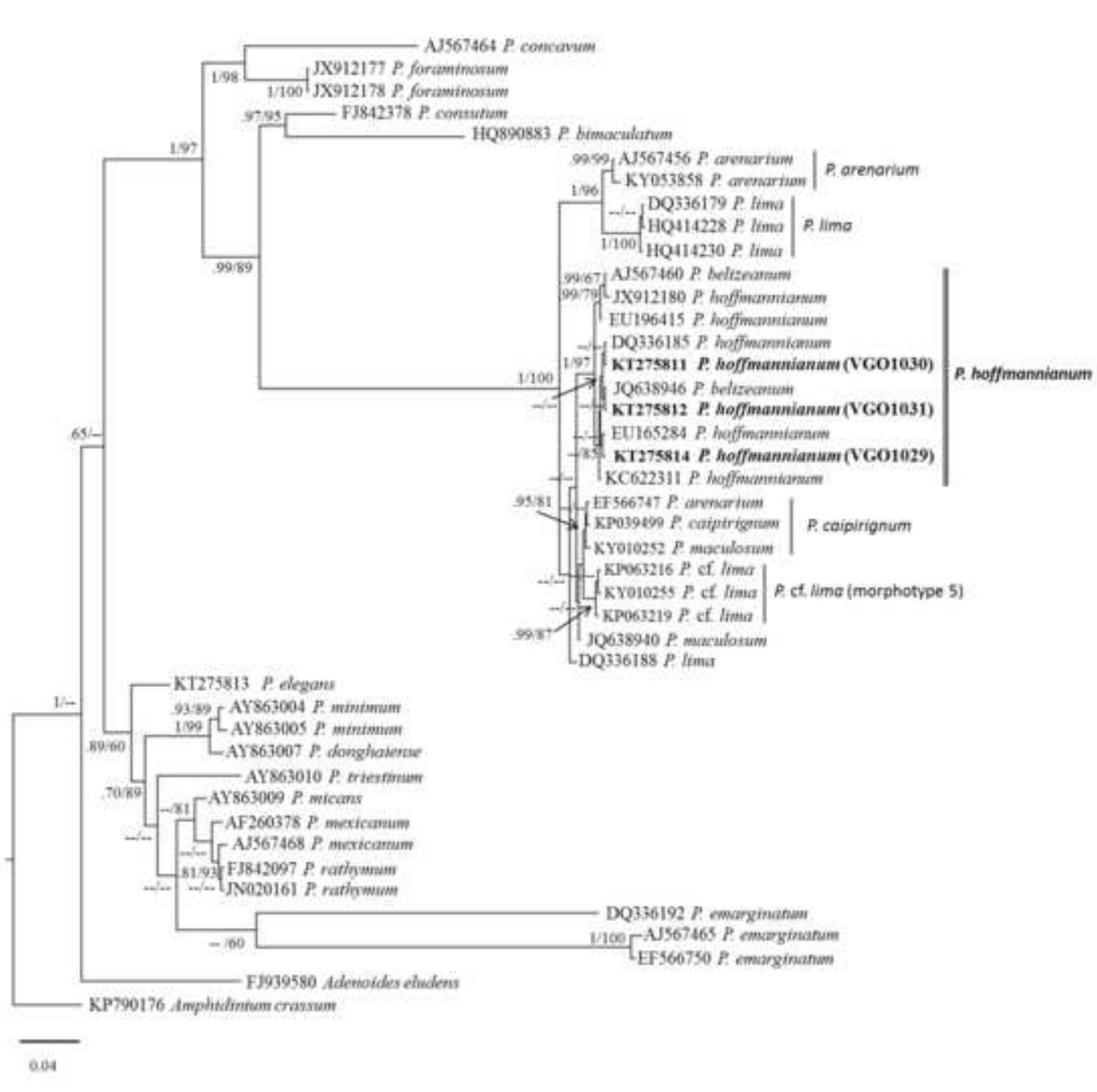

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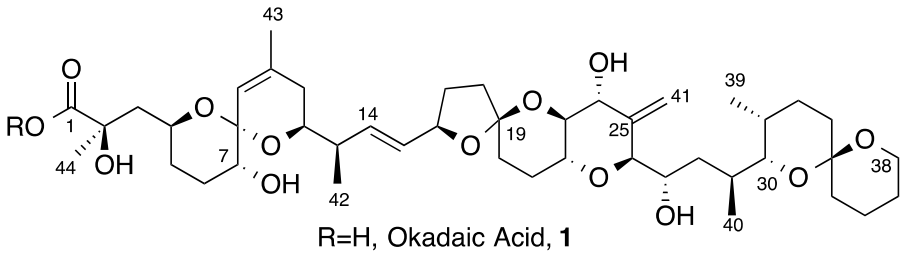
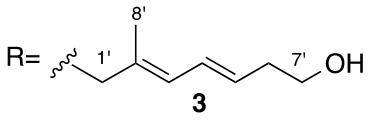


R=H, Okadaic Acid, **1**



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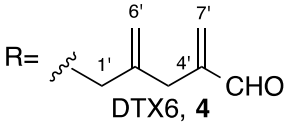
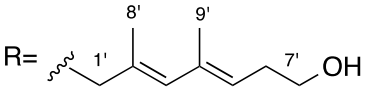


DTX6, **4**



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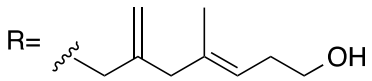

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Table

Table 1. DSP toxins detected in *Prorocentrum hoffmannianum* from Canary Islands.

Strain			VGO1029		VGO1030		VG01031	
Compound	Molecular formula	RT (min)	Cells	Medium	Cells	Medium	Cells	Medium
1	$C_{44}H_{68}O_{13}$	3,7	+	+	+	+	+	+
2	$C_{44}H_{68}O_{12}$	5,23	+	-	+	+	+	-
3	$C_{52}H_{80}O_{14}$	7,3	+	+	+	+	+	+
4	$C_{51}H_{76}O_{14}$	7,4	+	-	+	-	+	-
5	$C_{53}H_{82}O_{14}$	7,5	-	+	-	+	-	+
6	$C_{53}H_{82}O_{14}$	7,6	-	+	-	+	-	-