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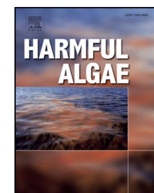

The toxic benthic dinoflagellate *Prorocentrum maculosum* Faust is a synonym of *Prorocentrum hoffmannianum* Faust

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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.

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, 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

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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 et al., 1996; Cruz et al., 2006; Paz et al., 2007b; aand 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

2.1. Source of specimens and culture conditions

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

2.2. Light microscopy

Cultured cells were observed under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with differential interference contrast. Naming of morphological features followed the criteria of Hoppenrath et al. (2013).

2.3. Sample preparations for SEM

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 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′-ACCCGCTGA ATTTAAGCATA-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 1000.000. 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.

2.6. Culture of prorocentrum strains for toxin analysis

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

2.7. Toxin extraction

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

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