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Notes on *Ostreopsis* sp. from southern-central coast of Cuba

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Abstract – In order to investigate the identity, abundance and toxicity of a distinctive *Ostreopsis* species present in the southern-central coast of Cuba, *Ostreopsis* field populations were examined during January-September of 2010. The morphological and partial LSU phylogenetic data suggested the presence of *O. lenticularis*, although a very close species like *O. labens* could not be discarded. Results indicated the presence of palytoxin-like molecules in a natural extract at concentration of 0.12 pg/cell. *Ostreopsis* populations were very stable through the course of the study, and a maximum of 7.9×10^4 cells/g of macroalgae fresh weight was recorded on June.

Cuba / DNA sequences / *Ostreopsis* / palytoxin

INTRODUCTION

Benthic and epiphytic species of *Ostreopsis* Schmidt are widely distributed throughout the world and they are particularly important component of subtropical and tropical marine coral reef environments. Some *Ostreopsis* species produce palytoxin, one of the most potent marine biotoxins, or palytoxin analogues (Ciminiello *et al*., 2010, Lenoir *et al*., 2004, Rosi *et al.*, 2010).

In Cuba, despite some ecological studies on potentially toxic benthic dinoflagellates (Delgado *et al*., 2007), populations of *Ostreopsis* have not been investigated. High densities of a distinctive species of *Ostreopsis* have been observed during preliminary surveys of benthic dinoflagellates in shallow-sheltered lagoon reefs from southern-central coast of Cuba. The objective of this study was to characterize the temporal and spatial variability of *Ostreopsis* populations and provide a preliminary investigation on genetic and toxicity of *Ostreopsis* blooms in this Caribbean region.

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MATERIALS AND METHODS

Sample collection

The study area is an oligotrophic inshore coral reef, located 30 km offshore from Cienfuegos Bay on the southern-central coast of Cuba, Caribbean Sea. Sampling was conducted once monthly from January to September/2010. The concentration of *Ostreopsis* cells was estimated on *Dictyota* sp. Two independent samples of macroalgae with surrounding seawater were collected and placed in plastic bags. These were shaken vigorously and contents passed through a 250 μm, then 150 μm and finally 20 μm. This last fraction, retaining the dinoflagellates, was suspended in a known volume of filtered seawater and fixed with Lugol's solution. Cell enumeration was performed with a Sedgewick-Rafter chamber and numbers corrected to cells/g of macroalgae fresh weight (mfw). The typical cellular aggregates or mucilaginous layers described for other *Ostreopsis* species were not observed. Sea surface temperature and salinity were simultaneously measured.

Species identification

Species identification was based on the species original descriptions together with more recent descriptions. Field specimens were examined under a transmitted light microscopy *Leica* with phase contrast. Cells were observed alive or fixed with formalin. For plate pattern identification the cells were dissected squashing the cells by gently pressing the cover slip over them occasionally with the aid of sodium hypochlorite. More detailed characteristics, such as the thecal pores, were observed in scanning electron microscope (SEM).

DNA extraction

DNA extracts of *Ostreopsis* were used for amplification following a Chelex extraction procedure (Litaker *et al*., 2010). Cells of *Ostreopsis* were picked up with a micropipette, washed in three distilled water droplets, and placed in a 200 μL tube containing 10 μL of 10× PCR buffer. The tubes were centrifuged and 30 μL of 10% Chelex 100 (Bio-Rad, Hercules, California, USA) in dH_2O was added. The tubes were boiled at 95ºC in a Eppendorf Mastercycler EP5345 thermocycler (Eppendorf AG, New York, USA) for 10 min, then vortexed. The boiling and vortex steps were done twice and samples centrifuged (13,000 rpm for 1 min). The supernatants were transferred to clean 200 μL tubes and stored at – 20ºC until PCR amplification.

PCR amplification and DNA sequencing

The D1-D2 regions of the LSUrDNA were amplified using the pairs of primers D1R/D2C (Lenaers *et al*., 1989). The amplification reaction mixtures (25 μL) contained 4 mM $MgCl_2$, 0.5 pmol of each primer, 0.8 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, California, USA), and 2 μL from the single cell Chelex extractions. The DNA was amplified in a Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA) following the conditions detailed in (Litaker

et al., 2003), and reactions were checked by agarose gel electrophoresis The PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA), and sequenced using the Big Dye Terminator v3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) in a AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidade de Vigo, Spain). Genbank database do not accept sequences shorter than 200 bp, therefore the sequence from *Ostreopsis* in Cienfuegos is only available upon request to the authors.

Phylogenetic analyses

LSUrDNA sequences were inspected and aligned using CLUSTALW multiple alignment in Bioedit (Hall, 1999). The final alignments were converted to nexus files using SeqVerter 2.0 (GeneStudio, Inc., USA). The phylogenetic relationships were determined using a General Time Reversible model (GTR) in MrBayes v3.1 (Huelsenbeck and Ronquist, 2001). The program parameters were statefreqpr = dirichlet (1,1,1,1), nst = 6, rates = invgamma, nswaps = 1. Starting trees for each chain were selected randomly using the default values for the MrBayes program. The number of generations used in these analyses was 300,000.

Toxin analyses

Benthic dinoflagellates were collected in March 2011, from a natural sample (47.5 g of *Dyctiota* sp. fresh weight) with high concentration of *Ostreopsis* sp. (4.1×10^5 cells/g fwm in 650 ml of seawater with microalgal material). The water temperature during the bloom was 27.1°C. Then, seawater was filtered through GF/C filter (Whatman, Maidstone, UK). Toxin extraction from filter was performed with MeOH 100% in two steps obtaining 5 mL methanolic extract which was analysed by mouse bioassay (MBA), haemolytic assay (HA) and Liquid chromatography with fluorescence detection (LC-FLD).

Palytoxin activity was checked by both, MBA and HA. The MBA was performed following the method described by Riobó *et al.* (2008a) and the delayed hemolysis of sheep erythrocytes was analyzed following the method developed by Riobó *et al.,* (2008b). This assay depends on the ability of palytoxin to cause the hemolysis of red blood cells. The specific presence of palytoxin or its analogues is demonstrated by the prevention of hemolysis by including Ouabain like antagonist.

To chemically characterize the toxins, chromatographic analyses of the methanolic extract was performed by LC coupled to fluorescence detection following the pre-column derivatization method established by Riobó *et al.,* (2006) with slights modifications. Separations of extract and reference palytoxin (*Palythoa tuberculosa*, Wako Chemicals, Germany) were performed on Ascentis RP amide column, 5 µ (150 × 4.6 mm i.d.) at 35ºC. A linear gradient was applied between A (MeOH: Ammonium acetate 0.1 M, pH 4 (58:42) and B (MeOH) at a flow rate of 0.75 mL/min. Gradient elution from 60% B to 80% B was performed in 20 min, followed by a linear rise to 100% B in 5 min. Then, isocratic elution with 100% B was maintained for 3 min. Before new injections the system was returned to initial conditions in 2 min and equilibrated for 6 min.

RESULTS

Species identification

Cell is photosynthetic with many golden-brown chloroplasts. It has a plate pattern Po, 3', 7'', 5''', 2'''', 1 p. Cell size range from 60 to 85 µm in dorsoventral diameter and 50-67 µm in width. Cells varied from lenticulate to broadly oval, compressed anteroposteriorly and slightly pointed ventrally. The lack of undulation when viewed laterally was observed and only one type of thecal pore could be seen on surface (Fig. 1).

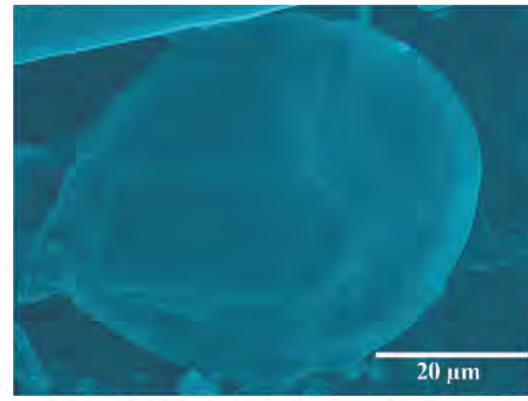

Fig. 1. Hypothecal view of *Ostreopsis* sp. in SEM.

Temporal pattern

Ostreopsis sp. was the most abundant benthic dinoflagellate (upper to 2.0×10^4 cells/g mfw) in the study area during all months. The temporal pattern of the abundance of *Ostreopsis* sp. is shown in Fig. 2. The maximum abundance (7.9×10^4 cells/g mfw) was recorded in June, while the minimum (2.0×10^4 cells/g mfw) was recorded in September. Other potentially toxic benthic species such as *Gambierdiscus caribaeus* (up to 1.6×10^3 cells/g mfw) and *Prorocentrum belizeanum* (up to 6.5×10^2 cells/g mfw) were well-represented in the benthic dinoflagellates community.

Salinity did not show large variation at the study area, the mean value was 35.1 psu. Water temperature ranged from 24.8°C on January to 30.7°C on September. Highest abundance of *Ostreopsis* sp. was recorded at higher water temperature.

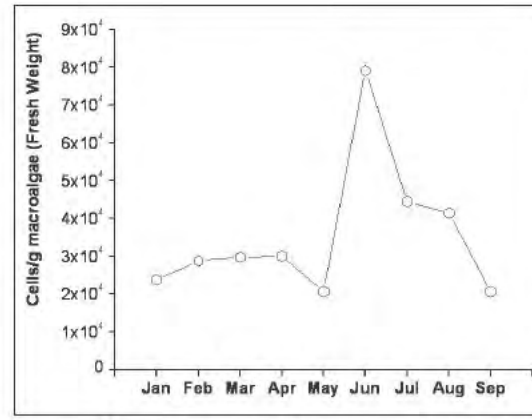

Fig. 2. Mean values of *Ostreopsis* sp. cells/g of *Dictyota* sp. fresh weight, Jan.-Sept./2010.

Genetic analyses

The LSU phylogeny (D1-D2 region) grouped the sequence obtained for *Ostreopsis* isolated in Cienfuegos with a sequence of *O. lenticularis* from Malaysia (Pin *et al.* 2001) (Fig. 3). The short amplified fragment (only 115 bases) prevented a more robust phylogeny. However, given the high similarity relative to

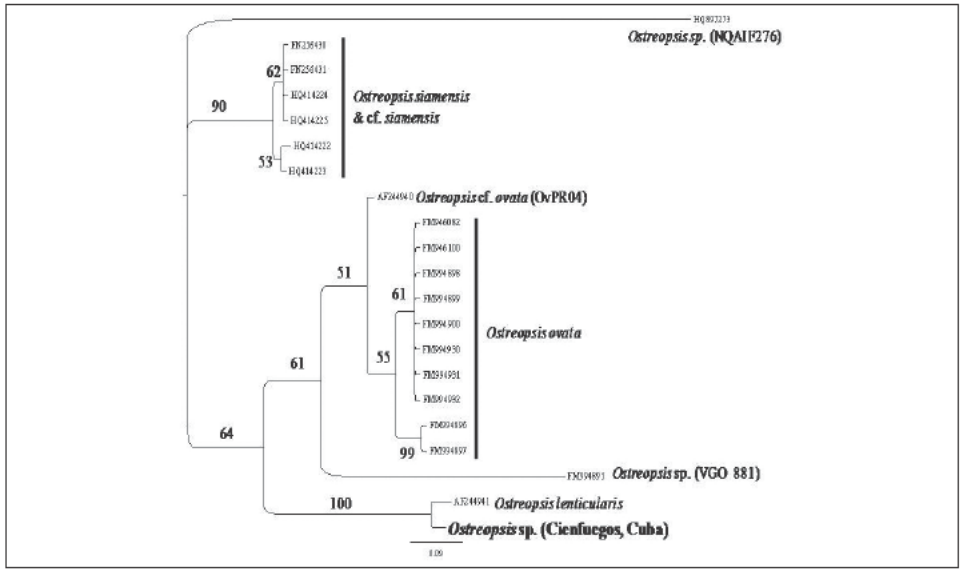

Fig. 3. LSU phylogeny (D1-D2 region) showing the relationship between the *Ostreopsis* sequence obtained from single cell isolates in Cienfuegos and other *Ostreopsis* species. The GenBank accession numbers for the isolates included in the analyses are shown in the phylogenetic tree. Supports at internal nodes are posterior probability values (Bayesian analyses).

O. lenticularis (0.95) and its divergence relative to other species, we suggest that the sequence obtained in Cienfuegos probably belongs to *O. lenticularis*, or a very close genetic species.

Toxins

The bloom is toxic. Epiphytic samples taken during the bloom period showed typical haemolytic activity due to palytoxin which was inhibited in presence of ouabain. Moreover, two mice were i.p. injected with the *Ostreopsis* extract. Mice dead between the 4 and 24 hours after injection showing the typical symptoms related with palytoxin (see Riobó *et al.*, 2008a). Presence of palytoxin was also determined by LC-FLD (Fig. 4). The toxin content in cells from field sample has been estimated to be 0.12 pg palytoxin equivalents/cell by both, HA and LC-FLD.

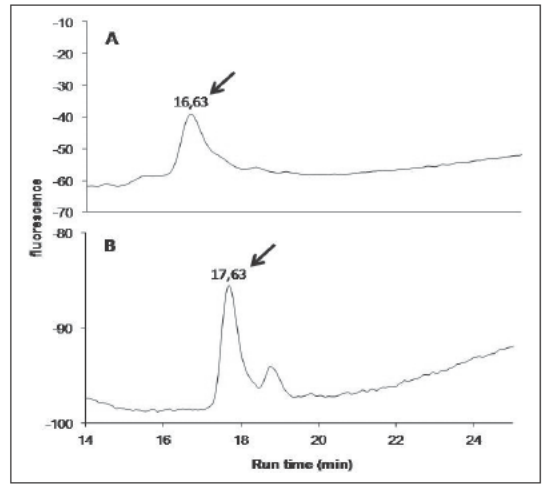

Fig. 4. Representative chromatograms of derivatized palytoxin standard (A) and derivatized *Ostreopsis* extract (B). The arrow indicates the palytoxin peak.

DISCUSSION

High abundance of *Ostreopsis* in some geographical areas from the Caribbean has previously been reported. *O. lenticularis* is the principal component of the epiphytic benthic microflora on macroalgae in shallow coral reef environments in the Puerto Rico and Virgin Islands (Ballantine *et al*., 1985; Carlson & Tindall, 1985). The density of *Ostreopsis* sp. presented here was clearly stable and high during all study. On the contrary, in southwestern Puerto Rico, populations of *O. lenticularis* were highly variable in density (Ballantine *et al*., 1985).

Identification of the *Ostreopsis* species based on morphological characters is very difficult since plate pattern, size and thecal surface is quite similar in several *Ostreopsis* species. In the present study, the morphology of *Ostreopsis* fitted well with the existing taxonomical descriptions for *O. lenticularis* (Fukuyo, 1981; Faust *et al.,* 1996). The original description of *O. lenticularis* by Fukuyo (1981) reported the presence of fine pores in addition to the thicker trichocyst pores seen in other *Ostreopsis* species. However, our SEM pictures were not resolutive enough to inspect these fine pores, but given the genetic distances in our analyses, we can discard that it could belong to *O. ovata*, *O. siamensis* or *Ostreopsis* sp. (VGO 881) isolated from the Canary Islands.

Characterization of *Ostreopsis* species in the Caribbean islands has traditionally been done only by morphology but it is known that phylogenetic analyses in *Ostreopsis* have been helpful in determining species identifications and geographic distribution studies (Penna *et al*., 2010). Based on the morphological and phylogenetic results of *Ostreopsis* in this study we might suggest that *O. lenticularis* was the species present, however, because of the ambiguities concerning similar *Ostreopsis* species we suggest it is more reasonable to name *Ostreopsis* sp. until more taxonomical characterization is available.

Further studies in the area should include not only larger LSUrRNA sequences (full D1/D2 domain) but other more resolutive molecular markers as the internal transcribed spacer (ITS) regions. This is particularly important in our case because *O. lenticularis* and *O. labens* share very similar ITS sequences (Penna *et al*., 2010). Thus, prior to confirm the identity of *Ostreopsis lenticularis* or *O. labens* in the study area, further morphological and genetic studies would be required to confirm the differences between both species.

Toxin analyses performed in this study showed the presence of palytoxin-like compounds (0.12 pg/cell) in the *Ostreopsis* extract. Aligizaki *et al*. (2008) reported higher values (0.3-0.7 pg/cell) from mixed field *Ostreopsis* spp. in Greek coasts; and Lenoir *et al*. (2004) reported lower values (0.04 pg/cell) for *O. mascarensis* in field bloom from Rodrigues Island, Indian Ocean.

In the Caribbean (Puerto Rico and Virgin Islands), previous findings from cultured *O. lenticularis* have demonstrated toxicity only through biological assays (Ashton *et al*., 2003; Tindall *et al*., 1990). According to our information, the present study could be the first report of palytoxin-like compounds in this species, determined for both bioassays and chemical analyses.

It should be pointed out that the presence of abundant toxic *Ostreopsis* sp. populations constitute a potential threat for human health in the central-southern region of Cuba since palytoxin like compounds have been associated to poisonings by different exposure routes such as oral exposure after contaminated seafood consumption or inhalational and cutaneous exposures to aerosolized seawater during *Ostreopsis* blooms (Tubaro *et al*., 2011).

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