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Contribution to the genus *Ostreopsis* in Reunion Island (Indian Ocean): molecular, morphologic and toxicity characterization

Olga CARNICER[a], Alina TUNIN-LEY[b], Karl B. ANDREE[a], Jean TURQUET[b], Jorge DIOGÈNE[a] & Margarita FERNÁNDEZ-TEJEDOR[a,b]*

[a] *IRTA, Carretera de Poble Nou, km 5.5, 43540 Sant Carles de la Ràpita, Spain*

[b] *ARVAM, Agence pour la Recherche et la Valorisation Marines, F-97490 Sainte Clotilde, France*

Résumé – Les dinoflagellés épibenthiques et toxiques du genre *Ostreopsis* sont rencontrés dans tous les écosystèmes coralliens tropicaux et les régions tempérées. Jusqu'ici, neuf espèces morphologiques ont été décrites dans ce genre. Certaines produisent des composés de type palytoxine et présentent un danger pour les organismes marins et pour la santé humaine. Les espèces étant très ressemblantes au niveau morphologique, la taxonomie du genre est actuellement sujette à révision. Cette étude vise à fournir de nouvelles données concernant la morphologie, la phylogénie et la toxicité du genre, à partir de l'analyse de trente-trois souches isolées sur la côte ouest de l'île de La Réunion (Océan Indien). Deux morphotypes de tailles distinctes ont pu être identifiés : un petit morphotype (DV = 53,5 ± 6,9 µm ; W = 37.7 ± 5,6 µm) présentant une forme de goutte typique, et un grand morphotype (DV = 103,9 ± 5,1 µm ; W = 85,3 ± 6,9 µm) avec une forme très arrondie. L'analyse phylogénétique a révélé l'existence de trois espèces distinctes. Le petit morphotype correspond à deux espèces, *O.* cf. *ovata*, et une espèce cryptique jamais caractérisée auparavant. Le grand morphotype constitue un clade distinct et génétiquement homogène. La faible différence en nucléotides ($p < 0{,}088$) entre cette espèce et celle qualifiée par Sato *et al.*, 2011 d'*Ostreopsis* sp. 5, suggère que les deux souches constituent une même espèce. Les tests hémolytiques n'ont montré aucune activité de type palytoxine pour les trois espèces.

***Ostreopsis* / taxonomie / phylogénie / morphologie / composés de type palytoxine**

Abstract – The toxic epi-benthic dinoflagellate *Ostreopsis* is distributed worldwide in coral reef ecosystems and temperate regions. There are nine species described to date based on morphological features. Some of them have been proved to be producers of palytoxin-like compounds, representing a threat to coastal marine organisms and human health. The taxonomy of the genus is currently under revision due to morphological similarities among species. The present study aims to provide additional information on morphology, 5.8S and ITS data and toxin content from thirty three strains isolated along the west coast of Reunion Island, in the Indian Ocean. Two morphotypes, non overlapping in size, were distinguishable: the small morphotype (DV = 53.5 ± 6.9 µm; W = 37.7 ± 5.6 µm) with a typical tear-drop shape and the large morphotype (DV = 103.9 ± 5.1 µm; W = 85.3 ± 6.9 µm) with a rounded shape. Phylogenetic analysis revealed the presence of three genotypes. Within the small morphotype, two different species were identified, *O.* cf. *ovata* and a cryptic species not previously characterized. The larger cells constituted a genetically

* Corresponding author: Margarita Fernández-Tejedor (margarita.fernandez@irta.cat)

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homogeneous clade. Nucleotide divergence between this species and the one qualified by Sato *et al*., 2011 of *Ostreopsis* sp. 5 was relatively low ($p < 0.088$) and those two strains are likely to be the same species. Haemolytic analysis resulted in no palytoxin-like activity in any of the three species.

***Ostreopsis* / taxonomy / phylogeny / morphology / palytoxin-like compounds**

INTRODUCTION

The dinoflagellate *Ostreopsis* spp. grows in tropical and temperate epibenthic microalgae communities and has been described within the benthic dinoflagellate communities in ciguatera endemic areas (Tindall *et al.*, 1984). This genus has a wide distributional range, though the majority of the described species have been found in tropical waters. The nine species identified so far are characterised based on morphological features, *O. siamensis* Schmidt 1901; *O. ovata* Fukuyo 1981; *O. lenticularis* Fukuyo 1981; *O. heptagona* Norris *et al.,* 1985; *O. mascarenensis* Quod 1994; *O. labens* Faust & Morton, 1995; *O. marinus* Faust, 1999; *O. belizeanus* Faust, 1999 and *O. caribbeanus* Faust, 1999. However, the taxonomy of the genus needs a thorough revision since different species with the same morphological characteristics show significant genetic divergence. Plate pattern is very similar for all species of the genus with the exception of *O. heptagona* (Faust *et al.,* 1996). Variability in size within the same species has been observed both in field samples (e.g. Aligizaki & Nikolaidis*,* 2006; Accoroni *et al.,* 2012; Carnicer *et al.*, unpublished data) and in cultures (Guerrini *et al.,* 2010; Pezzolessi *et al.,* 2012; Bravo *et al.,* 2012), adding more difficulty to differentiate the species. Recent studies (Pin *et al.,* 2001; Penna *et al.,* 2005; Penna *et al.,* 2010; Laza-Martínez *et al.,* 2011; Sato *et al.,* 2011; Kang *et al.,* 2013; David *et al.,* 2013) have implemented molecular taxonomic analysis using the SSU, LSU, 5.8S, ITS1 and ITS2 sequences of the ribosomal DNA operon in addition to the morphological descriptions, to counteract this problem and clarify the present problematic taxonomy.

During the last decade, proliferations of *Ostreopsis* spp. along the Mediterranean coasts have been reported and associated with several episodes of human intoxication (reviewed in Tubaro *et al.,* 2011). Blooms occurring in summer-fall months are produced by *O.* cf. *ovata* in most cases, or together with *O.* cf. *siamensis* in lower abundance (Mangialajo *et al.,* 2011). These recurrent proliferations associated with human intoxication have motivated many studies on these two species and have brought new insights on the ecology, toxicity and phylogeny of both species in the Mediterranean Sea. Nonetheless, tropical species of *Ostreopsis* remain poorly studied though the species diversity of *Ostreopsis* is higher in tropical latitudes than in temperate areas. Recent studies on the taxonomy of the dinoflagellate genus *Gambierdiscus* Adachi R. & Fukuyo Y., 1979, another benthic taxon co-occuring with *Ostreopsis* in tropical areas, have revealed that the genus *Gambierdiscus* exhibits remarkable diversity with both endemic and cosmopolitan taxa (Litaker *et al.,* 2010, Nishimura *et al.*, 2013); whereas former studies mostly reported a single species, *G. toxicus,* which was erroneously consider as a widespread species (Richlen *et al.*, 2008). Likewise, the genus *Ostreopsis* may hold cryptic genetic diversity as *Gambierdiscus* and it may be more complex than is currently suggested by the morphological descriptions.

More widely, molecular tools have highlighted, within benthic dinoflagellates, a larger diversity than previously reported, leading to the descriptions of many new species and genera (Litaker *et al.,* 2007). These results should motivate a deeper investigation of the actual species diversity within the genus *Ostreopsis* considering both genetic and morphological characters. The nuclear rDNA internal transcribed spacer regions (ITS1 and ITS2) and 5.8S rRNA gene have been used in multiple phylogenetic studies (e. g. Jensen *et al.,* 1993, Hartmann *et al.,* 2001) and represent a useful molecular marker to characterize *Ostreopsis* (Penna *et al.,* 2005). Unfortunately, there is no genetic material for the nine type species of *Ostreopsis* described so far from a morphological basis, it is therefore not possible to assign sequences obtained in new studies to a particular species of the genus with full confidence. For instance, as a cautionary measure taken after the first phylogenetic studies, the species found in the Mediterranean Sea (the most studied so far) are named, pending confirmation, *O.* cf. *siamensis* (Penna *et al.,* 2005) and *O.* cf. *ovata* (Penna *et al.,* 2010). In recent years, studies have provided new sequences from *Ostreopsis* cells isolated in tropical waters increasing the possibilities of matching described morphologies with genetic material. Sequences from the Pacific, the eastern Indian, the western and eastern Atlantic Oceans, have been added to the numerous sequences available from the Mediterranean Sea isolates in GenBank, contributing to a better understanding of the phylogenetic relationships among species. Furthermore, toxin composition is an additional characteristic that may contribute to assignation of a species-specific toxin profile (Penna *et al.,* 2005). Various species belonging to the genus *Ostreopsis* have been identified as palytoxin (PLTX)-like compound producers; Ostreocin-D (Usami *et al.,* 1995) from *O. siamensis*; mascarenotoxins from *O. mascarenensis* (Lenoir *et al.,* 2004); putative PLTX and ovatoxins analogues from *O.* cf. *ovata* (Ciminiello *et al.,* 2012). Discrimination between toxic and non-toxic species has been observed in other toxic dinoflagellate genera such as *Alexandrium* (Yoshida *et al.,* 2001) and *Gambierdiscus* (Litaker *et al.,* 2010). Therefore, a preliminary toxicity analysis would contribute to the characterization of new species identification.

In the present study we conducted a comprehensive sampling of the genus *Ostreopsis* in the Reunion Island, western Indian Ocean with the aim to assess genetics, morphology and toxicity diversity of *Ostreopsis* strains from the south-west Indian Ocean where *O. mascarenensis*, *O. siamensis*, *O. ovata*, *O. lenticularis, O. labens,* (Faust *et al*., 1996), and *O. marinus* (Faust, 1999) have been reported.

MATERIAL AND METHODS

Sampling strategy and culture conditions

Sampling sites were located along the east coral reef coast in Reunion Island (Fig. 1) and samples were collected weekly from September to November 2013. Different species of macroalgae were collected in each site, *Actinotrichia fragilis* (Forsskål) Børgesen 1932*, Turbinaria conoides* (J. Agardh) Kützing 1860*, Jania* sp. (J. V. Lamouroux, 1812) and *Galaxaura* sp. (J. V. Lamouroux, 1812) between

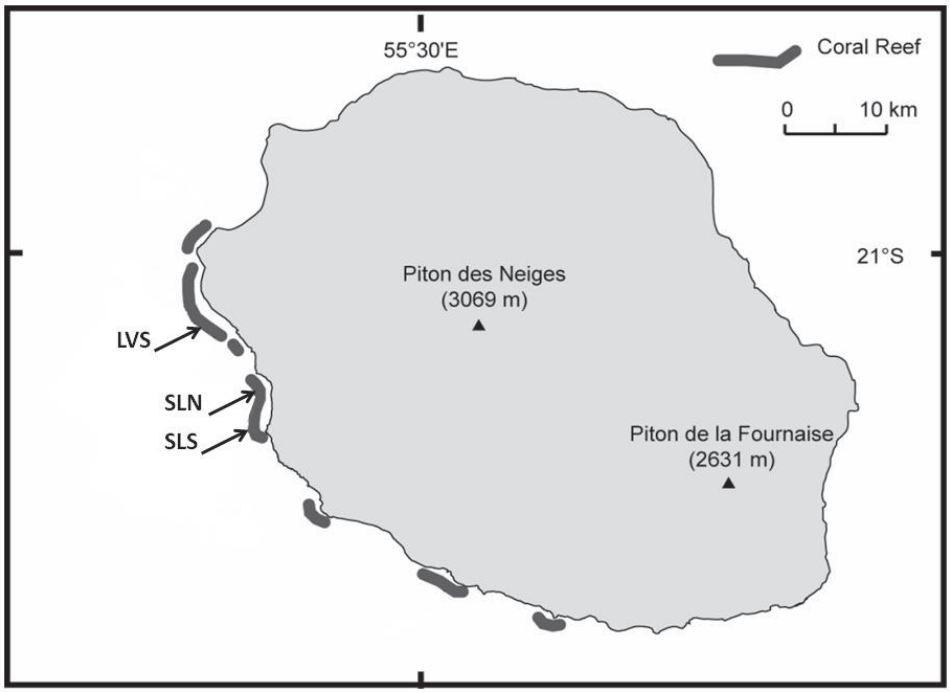


Fig. 1. Geographic location of the studied sites in La Réunion, West Indian Ocean.
Location acronyms and positions: LVS, Livingstone -21º 5' 51.3276''N; 55º 14' 17.6568'' E; SLN, Saint-Leu Nord -21º 10' 52.971''N; 55º 17' 12.9516'' E; SLS, Saint-Leu Sud, -21º 10' 52.971'' N; 55.286931E

1.5 and 0.5 meters depth. Macroalgae were placed in a plastic bottle with 200 mL of 0.2 µm filtered seawater. In order to release the epiphyte community from macroalgae, bottles were intensively shaken for one minute and filtered through a 200 µm mesh so that particles were eliminated. Once in the laboratory, *Ostreopsis* cells were isolated with a glass pipette by capillary method (Hoshaw & Rosowski, 1973) under an inverted microscope (Olympus CK2). Cells were transferred to a 15-well culture microplates (FALCON) filled with autoclaved filtered natural seawater containing f/2 concentrated nutrients diluted five-fold (Guillard, 1975). Cultures were maintained at 26°C under 12:12h light:dark photoperiod. Illumination was provided by fluorescent tubes with a photon irradiance around 20-40 µmol photons $m^{-2} \cdot s^{-1}$. After an initial growth, cultures were inoculated in 25 cm^2 glass Erlenmeyer flasks filled with 50 mL of medium. At the end of the exponential phase, cultures were inoculated to 150 cm^2 glass Erlenmeyer flasks filled with 450 mL of acclimated medium for three weeks until cells were filtered for toxin extraction.

A total of thirty three *Ostreopsis* strains were isolated and DNA extractions were prepared. These extracts were later used for genetic characterization (see below). Of the total strain collection, twenty seven strains survived after three weeks in culture. Methanol extraction was performed for a subsequent toxin analysis.

Morphological observation

Morphology was described from cells obtained in fixed field samples to avoid reporting possible deformations common in *Ostreopsis* spp. cells grown in culture. Samples, preserved with acidic lugol were analyzed under a light microscope (NIKON eclipse 80i) after staining with fluorescent Calcofluor White M2R, based on Fritz and Triemer (1985) methodology. Pictures in white light and in epifluorescence were taken using digital cameras © NIKON Digital Sight DS-5Mc and © HAMAMTSU ORCA-ER, respectively. To suppress focus problems and provide the most of morphological details, several images of each cell were taken and assembled using © Helicon Focus software.

Molecular characterization

The DNA extraction protocol followed Andree *et al.,* (2011). Polymerase chain reaction (PCR) conditions and primers ITSA and ITSB were those used in Sato *et al.,* (2011). ITS and 5.8S ribosomal RNA (rRNA) regions were amplified in an Applied Biosytems, 2720 Thermal cycler. Resulting fragments of 350-base pair (bp) rRNA were evaluated by electrophoresis in agarose gel (1.5% wt/vol) stained with GelRed™ (Biotium Inc., Hayward, California, USA) and were sent to be sequenced bidirectionally (GENOSCREEN; Paris, France) using the same primers as those used in the initial amplification. Forward and reverse sequence reactions were aligned and manually edited using BioEdit, version 7.0.0 (Hall, T.A., 1999). Genetic distances were obtained by Kimura's two-parameter model (Kimura, 1980) with MEGA 5.1.

When several strains shared the same sequence, only one strain was included in the phylogenetic study. Multiple analytical approaches were utilized including, Maximum likelihood (ML) and Neighbor-joining (NJ) using Kimura's two-parameter model (Kimura, 1980) or Tamura-3 model (Tamura, 1992) with MEGA 5.1. The sequences of *Coolia monotis* VGO783, CM01 and IEO-CM7C were initially included as outgroups for the 5.8S-ITS ribosomal gene phylogeny however these outgroup species were removed because they were too divergent to properly align the most probable homologs, as also found in Sato *et al*., 2011, and therefore we present an unrooted tree with no outgroup.

To estimate evolutionary divergence between ITS rDNA sequences we performed a pair-wise comparison, using the Kimura 2-parameter model (Kimura, 1980), to calculate the number of base substitutions per site between sequences.

Toxin extraction

Twenty seven cultures of different strains were extracted for toxin evaluation after twenty-one days of growth. Cells were collected by filtration on 0.45 µm nylon filters and were stored at –80°C. For toxin extraction, 15 mL methanol/water (80:20) solution was added to filters and these were sonicated for 5 minutes in pulse mode (Touch-Screen Sonicator Ultrasonic Processor, Qsonica, LLC). The mixture was centrifuged at 600 x g for 10 minutes; the supernatant was decanted and filtered through polytetrafluoroethylene 0.45 µm membrane syringe filters. This procedure was repeated twice and filtered supernatants were pooled. The final extract was evaporated to a 25 mL methanol/water (80:20) final volume.

Haemolytic assay

The haemolytic test was performed following the method of Riobó *et al.,* (2008) with some modifications in reactive solution concentrations in order to perform a valid calibration curve. Palytoxin and PLTX-like analogs bind to the Na^+/K^+ pump provoking cell lysis and the heamoglobin released is quantified afterwards using a microplate reader, model KC4 from BIO-TEK Instruments, Inc. (Vermont), at 405 nm absorbance. A calibration curve was prepared by using PLTX standard (Wako Chemicals GmbH, Germany) with 12 concentrations from 12.5 to 1250 $pg \cdot mL^{-1}$ adjusted to an exponential regression using SigmaPlot 9.0.

Working solution was prepared with washed sheep blood (OXOID), centrifuged (400 g, 10°C, 10 min) twice and diluted with phosphate buffered saline solution (PBS) 0.01 M, pH 7.4 (Sigma), 0.1% bovine serum albumin (BSA), 1 mM calcium chloride ($CaCl_2$ $2H_2O$) and 1 mM boric acid (H_3BO_3) to a final erythrocyte concentration of 1.5×10^6 $cells \cdot mL^{-1}$. The PLTX assay specificity is verified by a blank assay with ouabain (final concentration of 2 mM). Ouabain is a glycoside that prevents PLTX binding to Na^+/K^+ pumps and thus, inhibits haemolytic activity. Toxin extractions and PLTX standard were evaporated and refilled with PBS solution to eliminate methanol and water from the extraction. Assay was performed in two non-treated 96 well microplates and samples were settled in triplicate. After 22 hours incubation at 24ºC, microplates were centrifuged (200x g, 10 min), 200 µL of the supernatant was transferred to another microplate for absorbance reading. Total toxicity was expressed as PLTX equivalents per milliliter (PLTX $eq \cdot mL^{-1}$).

RESULTS

Description of strain isolation conditions such as location, date, macroalgae sampled, temperature and salinity are detailed in Table 1. *Ostreopsis* spp. abundances during sampling were very low (maximum of 200 $cell \cdot g\ fwm^{-1}$), with the small morphotype being dominant.

Morphological observations

Isolated cell morphology corresponded to the description of the genus *Ostreopsis*; photosynthetic, anterioposteriorly compressed and ventrally pointed cells. Plate pattern was Po, 3´, 7´´, 5´´´, 2´´´´, 1p. Two different cell morphologies were found in field samples, a group of large rounded cells and another one with a tear-drop shape and smaller size. Dorsoventral (DV) and width (W) diameter measurements from 73 cells from field samples are represented in Figure 2. The large cells displayed a broad ovoid shape very similar to *O. lenticularis* nevertheless the size range (DV=103.9±5.1 µm; W=85.3±6.9 µm; n=40) fit more closely with *O. siamensis* (Faust *et al.,* 1996) and *O. marinus* (Faust, 1999). The epifluorescence microscopy revealed a plate arrangement similar to *O. marinus* (Faust, 1999) with the presence of large pores and small pores or depressions (discernible at x600 magnification), randomly distributed on the thecal surface (Figures 3-9). This latter feature seems to coincide with the SEM observations of pores of different sizes in *O. siamensis* (Faust *et al.,* 1996). The apical pore is long

Table 1. Description of *Ostreopsis* strains collected in Reunion Island; location, date, macroalgae specie, temperature and salinity (nd = no data; * = 21º 11' 58.9092''N; 55º 16' 56.9706'E; ** = 21o 16' 10.2"N; 55º 19' 58.6632" E)

			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*
	1	P-0117																														
	2	P-0128	**0.010**																													
	3	AF218459	**0.005**	**0.005**																												
O. cf. *ovata*	4	AB674904	**0.005**	**0.005**	0.000																											
	5	AF218460	**0.010**	**0.010**	0.005	0.005																										
	6	AF218463	**0.010**	**0.010**	0.005	0.005	0.010																									
	7	AF218455	**0.032**	**0.032**	0.026	0.026	0.032	0.032																								
	8	FM244724	**0.032**	**0.032**	0.026	0.026	0.032	0.032	0.016																							
	9	IRTA-SMM-11-09	**0.070**	**0.070**	0.065	0.065	0.070	0.065	0.065	0.070																						
	10	AB674902	**0.070**	**0.070**	0.065	0.065	0.070	0.065	0.065	0.070	0.000																					
	11	FM244736	**0.082**	**0.082**	0.076	0.076	0.082	0.082	0.065	0.070	0.043	0.043																				
	12	AB674911	0.154	0.154	0.148	0.148	0.154	0.148	0.154	0.154	0.136	0.136	0.136																			
Ostreopsis sp.1*	13	AB674909	0.161	0.161	0.154	0.154	0.161	0.154	0.161	0.161	0.142	0.142	0.142	0.005																		
	14	HE793379	0.167	0.167	0.160	0.160	0.167	0.160	0.167	0.167	0.141	0.141	0.154	0.070	0.065																	
Ostreopsis sp.2*	15	AB674913	0.174	0.174	0.167	0.167	0.167	0.167	0.167	0.167	0.187	0.187	0.173	0.200	0.193	0.186																
small morphotype	16	P-0132	0.266	0.259	0.259	0.259	0.267	0.259	0.260	0.275	0.242	0.242	0.257	0.303	0.295	0.287	0.250															
Ostreopsis sp.3*	17	AB674914	0.356	0.347	0.348	0.348	0.339	0.348	0.356	0.348	0.375	0.375	0.371	0.344	0.352	0.378	0.372	0.272														
O. cf. *siamensis*	18	AJ491335	0.306	0.300	0.299	0.299	0.307	0.299	0.315	0.334	0.283	0.283	0.299	0.314	0.313	0.333	0.296	0.291	0.405													
Ostreopsis sp.4*	19	AB674916	0.435	0.426	0.426	0.426	0.426	0.436	0.426	0.417	0.454	0.454	0.444	0.474	0.473	0.474	0.446	0.525	0.557	0.545												
	20	FM244728	0.409	0.400	0.409	0.409	0.409	0.409	0.382	0.400	0.391	0.391	0.383	0.428	0.428	0.464	0.408	0.454	0.524	0.475	0.465											
Ostreopsis sp.2**	21	JX987680	0.400	0.400	0.399	0.399	0.399	0.408	0.390	0.399	0.426	0.426	0.399	0.456	0.466	0.496	0.426	0.473	0.523	0.485	0.442	0.105										
	22	JX987674	0.408	0.408	0.408	0.408	0.408	0.408	0.381	0.399	0.390	0.390	0.390	0.446	0.447	0.474	0.426	0.445	5.523	0.446	0.474	0.048	0.059									

Table 1. Description of *Ostreopsis* strains collected in Reunion Island; location, date, macroalgae specie, temperature and salinity (nd = no data; * = 21º 11' 58.9092''N; 55º 16' 56.9706'E; ** = 21o 16' 10.2"N; 55º 19' 58.6632"E) *(continued)*

			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*
	23	AB674921	0.530	0.533	0.531	0.531	0.531	0.531	0.541	0.543	0.536	0.536	0.534	0.526	0.524	0.593	0.587	0.559	0.588	0.557	0.660	0.626	0.625	0.602								
Ostreopsis sp.6*	24	AB674920	0.550	0.553	0.551	0.551	0.551	0.551	0.562	0.563	0.536	0.536	0.526	0.538	0.536	0.576	0.595	0.555	0.610	0.536	0.658	0.601	0.613	0.602	0.021							
Ostreopsis sp.1**	25	AF218465	0.517	0.520	0.518	0.518	0.518	0.518	0.528	0.530	0.492	0.492	0.472	0.490	0.489	0.538	0.540	0.562	0.516	0.531	0.646	0.557	0.579	0.546	0.106	0.094						
	26	FM244728	0.516	0.518	0.517	0.517	0.517	0.517	0.527	0.528	0.490	0.490	0.470	0.500	0.499	0.549	0.550	0.585	0.527	0.530	0.646	0.567	0.600	0.567	0.124	0.112	0.016					
	27	AB674922	0.528	0.531	0.530	0.530	0.530	0.530	0.528	0.541	0.528	0.528	0.550	0.550	0.549	0.605	0.588	0.545	0.587	0.555	0.739	0.638	0.639	0.591	0.117	0.142	0.099	0.111				
	28	P-0108	0.663	0.655	0.667	0.667	0.667	0.667	0.695	0.682	0.655	0.655	0.674	0.667	0.679	0.742	0.763	0.607	0.709	0.603	0.705	0.599	0.612	0.606	0.372	0.363	0.339	0.354	0.380			
	29	AB674917	0.663	0.655	0.667	0.667	0.667	0.667	0.695	0.667	0.648	0.648	0.699	0.601	0.598	0.669	0.712	0.574	0.687	0.598	0.661	0.599	0.612	0.601	0.361	0.352	0.337	0.361	0.387	**0.065**		
Ostreopsis sp.5*	30	AB674918	0.663	0.655	0.667	0.667	0.667	0.667	0.695	0.667	0.648	0.648	0.699	0.601	0.598	0.669	0.712	0.574	0.687	0.598	0.661	0.599	0.612	0.601	0.361	0.352	0.337	0.361	0.387	**0.065**	0.000	
	31	AB674919	0.703	0.695	0.707	0.707	0.707	0.707	0.737	0.707	0.687	0.687	0.741	0.652	0.648	0.725	0.755	0.623	0.728	0.648	0.674	0.635	0.648	0.637	0.377	0.368	0.353	0.377	0.404	**0.088**	0.021	0.021

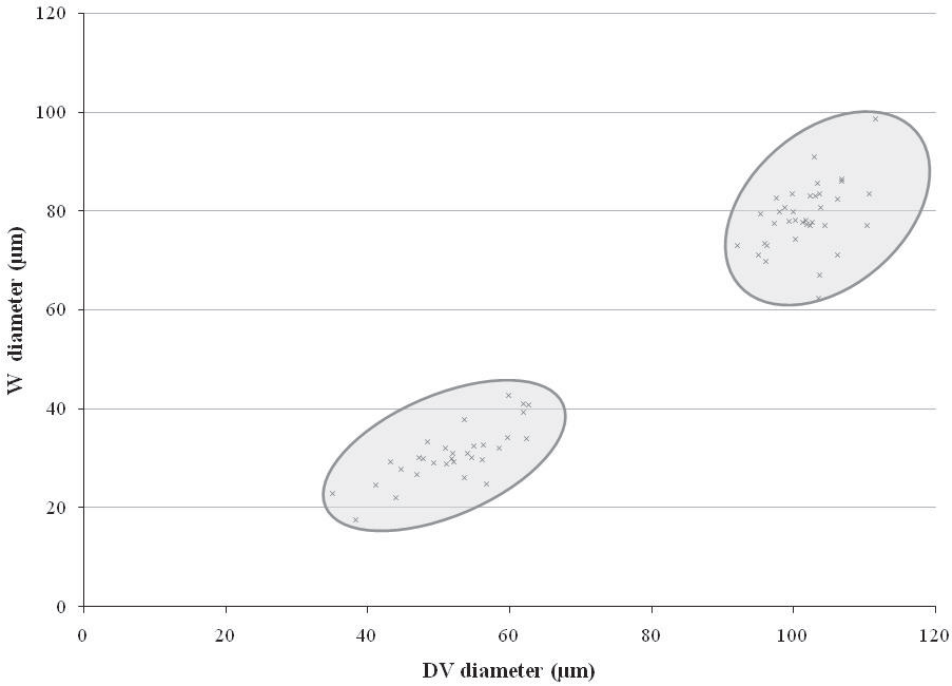


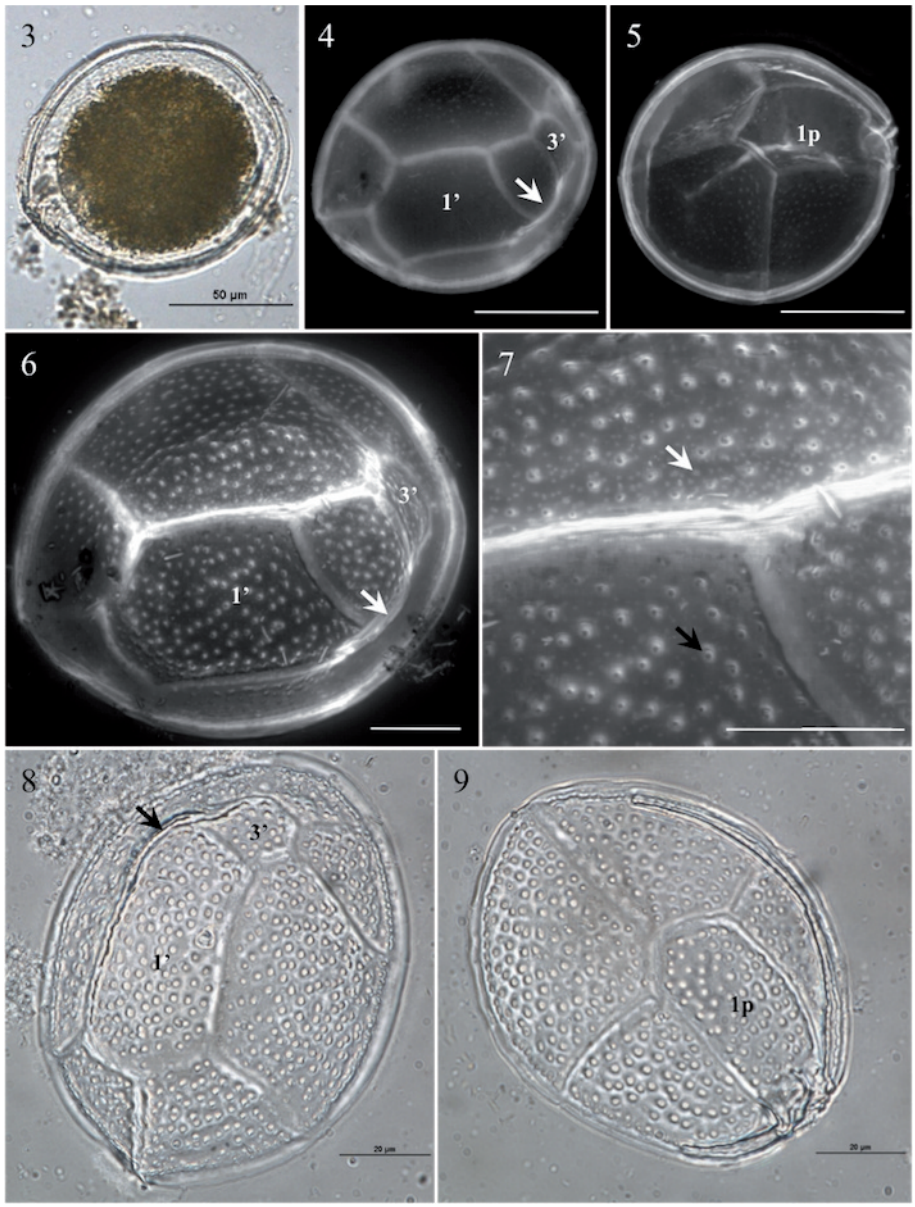
Fig. 2. Cell size, DV and W diameters (µm) of field cells, n = 73.

and curved like in *O. siamensis* and *O. lenticularis*. Based only on the morphological features, it was not possible to clearly identify the large morphotype.

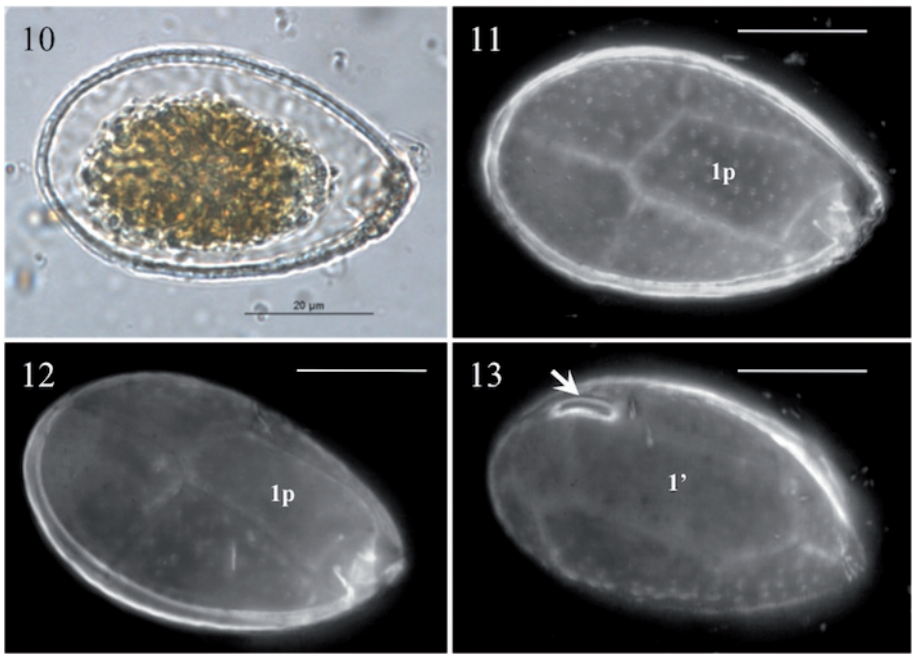
The small cells exhibited a tear-drop shape (DV = 53.5 ± 6.9 µm; W = 37.7 ± 5.6 µm; n = 33). Sizes mostly coincided to those reported for *O.* cf. *ovata (*reviewed in David *et al.,* 2013). The plate pattern observed with epifluorescence also resembled the plate arrangement in *O.* cf. *ovata*, along with the presence of pores (discernible at x600 magnification) evenly distributed on the thecal surface and a relatively straight apical pore (Figures 10-13). However, considering the fact that plate pattern is similar to all *Ostreopsis* species except *O. heptagona*, we cannot assure the correspondence to any particular species based upon morphological description alone.

ITS regions analysis

The PCR amplifications of 5.8S and ITS regions were obtained from thirty six cultured samples and the sequences obtained in this study have been deposited in GenBank under the accession numbers shown in Table 1. Those sequences were trimmed of primer sequences and aligned with forty four sequences from GenBank resulting in a final data set of 279 base pairs (bp). When using a rooted tree and *Coolia* sp. included as an outgroup there were difficulties in obtaining a clear consensus tree. However, nearly identical tree topologies,



Figs 3-9. Morphology of the "Large" morphotype in field samples and in cultures. **3-6.** Shape, plate arrangement of the theca in uncultured lugol-stained cells. The apical pore position is indicated by a white arrow. **7.** Ornamentation detail on the thecal surface of an uncultured cell, showing large pores (black arrow) and smaller pores or depressions (white arrow). **8-9.** Plate arrangement in empty cells from cultured strain P-0108. The apical pore position is indicated by a black arrow. **5, 9.** View of hypotheca. **3-4, 6-8.** View of epitheca. **3, 8, 9.** Bright Field microscopy images. **4-7.** Epifluorescence microscopy images with Calcofluor staining. Figs 3-5, bar scale = 50 µm. Figs 6-9, bar scale = 20 µm.



Figs 10-13. Morphology of the "Small" morphotype identified in field samples (lugol-stained cells). **10-12.** Shape and plate arrangement of the hypotheca. **13.** Shape and plate arrangement of the epitheca. The apical pore position is indicated by a white arrow. **10.** Bright Field microscopy images. **11-13.** Epifluorescence microscopy images with Calcofluor staining. Bar scale = 20 µm.

differentiating the same clades were obtained regardless of the analytical approach applied to the data when *Coolia* sp. was removed (Fig. 14).

Molecular diversity among Reunion Island and GenBank ITS rDNA sequences was evaluated using a pairwise comparison of nucleotide differences (Table 2). *Ostreopsis* cf. *ovata* isolated from Reunion Island had nucleotide substitution rate very low when compared with clades from the South China Sea (1% and with strains isolated from Malacca Strait and Indonesia (3.2%). Divergence was higher comparing strains from the Mediterranean clade (7%), KC17 from the Aegean Sea and VGO614 from Madeira (8.2%). The group of twenty nine isolates from Reunion Island is represented by P-0132 in Table 2, and had a nucleotide substitution value of 2.42 % when compared with the rest of the clades. The group of large cells that included five strains is represented by P-0108. Nucleotide differences were found to be low in a comparison with strains O70421.1 and O70421.2 (6.5%), and MB80828.4 (8.8%) from Japan. Phylogenetic analysis shows ten clades in accordance with other publications (Penna *et al.,* 2010; Sato *et al.,* 2011; David *et al.,* 2013). Five strains, coinciding with the group of cells with larger size reported in the morphologic analysis, P-0107; P-0108; P-0109; 79.1L and 79.2L (represented by P-0108 in Fig. 14), clustered very close to strains collected in Japan, in the Pacific Ocean, that were named as *Ostreopsis* sp. 5 in Sato *et al.,* (2011). From the group of small cells, two different genetic clades were distinguished. Two sequences corresponding to strains P-0117 and P-0128 were classified within the species *O.* cf. *ovata*, more precisely, in the Indo-Pacific clade, together with strains from the South China Sea and the South Pacific Ocean. The

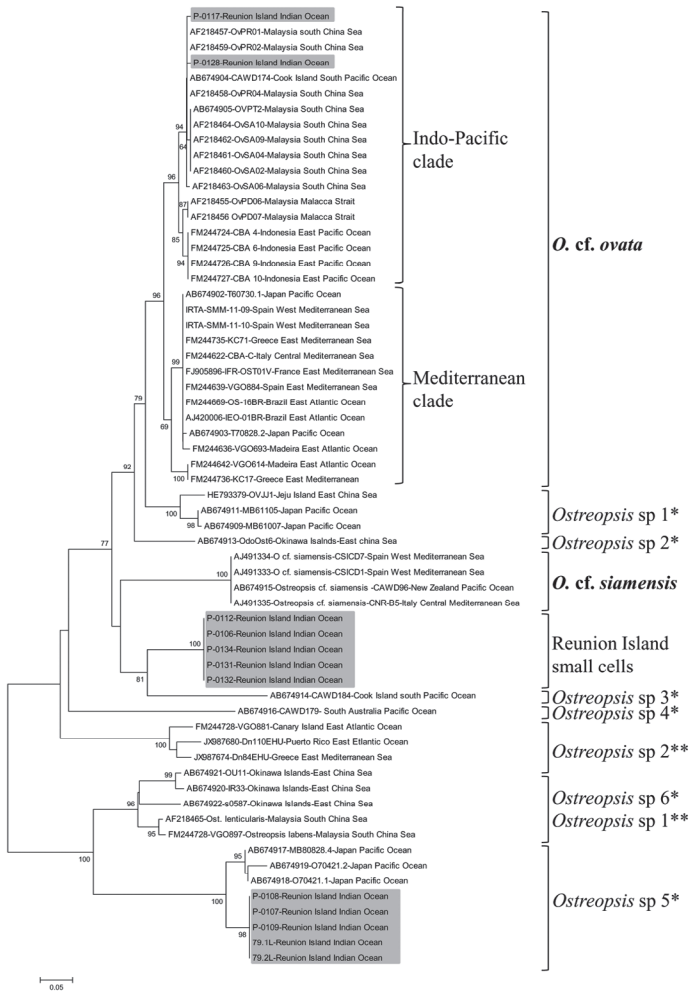


Fig. 14. Evolutionary relationships of taxa. The evolutionary history was inferred using N-J method. Bootstrap values (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

rest of the sequences of the small-sized cell, a total of 26, (represented by P-0132 in Figure 14), shared the same sequence, representing an independent clade that diverged from *Ostreopsis* sp. 3 (Sato *et al.,* 2011) collected in Cook Island, in the Pacific Ocean.

Toxin analysis

Haemolytic activity from the twenty seven strains isolated in Reunion Island was below the limit of detection (25 pg PLTX·mL^{-1}).

Table 2. Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model. Grey shading strains are those from Reunion Island. Values in bold could be considered as the same species for Reunion Island strains. * Described in Sato *et al*., 2011; ** Described in David *et al*., 2013.

ID	*Sampling point*	*Date*	*Macroalge*	*Temperature (°C)*	*Salinity*	*Accession Number*
P-0129	*	11/09/2013	n.d.	24.8	35.2	KM032205
P-0108	**	11/09/2013	Dead coral	nd	nd	KM032220
P-0111	SLN	18/09/2013	*Jania* sp.	24	34.9	KM032198
P-0130	SLS	18/09/2013	*Turbinaria conoides*	24.6	35.4	KM032192
P-0131	SLS	18/09/2013	*Turbinaria conoides*	24.6	35.4	KM032191
P-0132	LVS	18/09/2013	*Turbinaria conoides*	25.6	35.4	KM032190
P-0106	LVS	18/09/2013	n.d.	25.6	35.4	KM032206
P-0112	SLN	25/09/2013	*Turbinaria conoides*	23.8	35	KM032207
P-0133	SLN	25/09/2013	*Turbinaria conoides*	23.8	35	KM032194
P-0134	SLN	25/09/2013	*Turbinaria conoides*	23.8	35	KM032193
P-0107	SLN	25/09/2013	*Turbinaria conoides*	23.8	35	KM032217
P-0109	SLS	25/09/2013	*Turbinaria conoides*	23.8	35	KM032218
P-0104	LVS	25/09/2013	*Turbinaria conoides*	25.6	35.4	KM032204
P-0105	LVS	25/09/2013	*Turbinaria conoides*	25.6	35.4	KM032216
P-0102	LVS	08/10/2013	*Galaxaura* sp.	28.1	34.9	KM032209
P-0103	LVS	08/10/2013	*Galaxaura* sp.	28.1	34.9	KM032213
P-0117	LVS	16/10/2013	*Turbinaria conoides*	26	34.5	KM032202
P-0113	SLS	16/10/2013	*Turbinaria conoides*	24.6	35	KM032202
P-0114	SLS	16/10/2013	*Turbinaria conoides*	24.6	35	KM032201
P-0115	SLS	16/10/2013	*Turbinaria conoides*	24.6	35	KM032212
P-0116	SLS	16/10/2013	*Turbinaria conoides*	24.6	35	KM032203
P-0119	SLN	29/10/2013	*Galaxaura* sp.	25.3	33.2	KM032196
79.1L	SLN	29/10/2013	*Galaxaura* sp.	25.3	33.2	KM032221
79.2L	SLN	29/10/2013	*Galaxaura* sp.	25.3	33.2	KM032222
P-0121	SLN	29/10/2013	*Galaxaura* sp.	25.3	33.2	KM032219
P-0135	SLN	29/10/2013	*Galaxaura* sp.	25.3	33.2	KM032215
P-0122	SLN	29/10/2013	*Galaxaura* sp.	25.3	33.2	KM032211
P-0137	LVS	29/10/2013	*Turbinaria conoides*	27.2	34.9	KM032199
P-0126	LVS	29/10/2013	*Turbinaria conoides*	27.2	34.9	KM032197
P-0127	LVS	29/10/2013	*Jania* sp.	27.2	34.9	KM032214
P-0128	LVS	29/10/2013	*Jania* sp.	27.2	34.9	KM032210
P-0141	SLN	05/11/2013	*Turbinaria conoides*	26.6	34.4	KM032195
P-0142	SLS	05/11/2013	*Turbinaria conoides*	27.7	34.8	KM032200

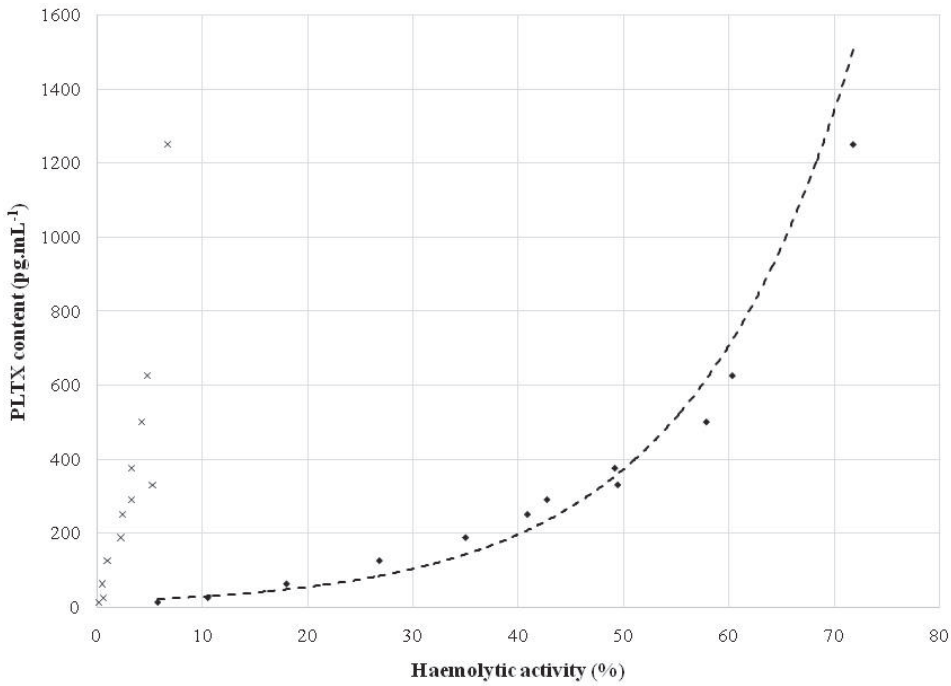


Fig. 15. Haemolytic assay exponential calibration curve without ouabain (dotted line) and with ouabain (crosses).

DISCUSSION

Observations from field samples, show that two morphotypes were identified based on size and shape differences. Plate pattern is almost similar among species (Penna *et al.,* 2005); it was therefore not possible to distinguish between species taking into account these criteria alone.

The large morphotype observed in our samples presented cell dimensions and an oval shape similar to the description of *Ostreopsis marinus* by Faust (1999) which actually was first described from coral debris collected in Mayotte Island, situated in SW Indian Ocean. We can thus postulate that the large cells observed in this study may be assigned to *O. marinus*.

Several species of *Ostreopsis* correspond to a similar morphological description. Further, as reported in David *et al.,* (2013) for *O.* cf. *ovata* and *O.* cf. *siamensis,* some species of *Ostreopsis* may overlap in cell sizes, which render difficult the use of these morphological features for identification. In the case of the small morphotype, no identification was possible based on light microscopy observations.

The use of molecular markers is recommended to untangle ambiguity in taxonomy based upon morphological characters, where both in the case of phenotypic plasticity and convergent morphology can mask true diversity. In this regard, phylogenies inferred from the ITS-5.8S rDNA differentiated the large

from the small morphotypes. Nevertheless, we still cannot be fully confident of our presumptive assignment to *O. marinus* as no sequences exist for the type species. In any case, this sequence is closely related to *Ostreopsis* sp. 5 (mean divergence = 7.26%) found in a previous phylogeographic study (Sato *et al.*, 2011). Interestingly, *Ostreopsis* sp. 5 was found near Japanese islands in the East China Sea, where temperatures registered were between 27 and 28°C, but was absent in the sampling performed in the same study in the north of Japan where the water was colder. Additionally, a later study regarding toxin content in Japanese strains analyzed by liquid chromatography did not detect PLTX-like compounds in *Ostreopsis* sp. 5, strain MB80828-3 (Suzuki *et al.,* 2012). In agreement to this result, Reunion Island toxin extractions of the five large morphotype strain cultures grown in the laboratory were below our limit of detection (25 pg $PLTX \cdot mL^{-1}$).

Within the small morphotype, two phylotypes corresponding to different species were identified by phylogenetic analysis; *O.* cf. *ovata* and another morphologically indistinct species. Many studies have been performed in the characterization of *O*. cf. *ovata* worldwide. There are two well-defined clades, from the Mediterranean Sea-Atlantic Ocean and from the Indo-Pacific Ocean (Penna *et al.,* 2010). In accordance with geographical distribution, Reunion Island *O.* cf. *ovata* strains were classified within the Indo-Pacific Ocean clade. In an early study among *Ostreopsis* populations in Malaysian waters (Pin *et al.,* 2001), two genetically-distinct groups were identified within this clade; one from Malacca Straits and another from the South China Sea. Contrarily to what might have been expected from geographical distribution, being that the Malacca Strait is open to the Indian Ocean, Reunion Island strains were genetically closer to those of the South China Sea clade. It can be hypothesized that genetic exchange with the Malacca Strait population is limited since it is separated by the Malaysian Peninsula from the Pacific Ocean (Pin *et al.,* 2001) and by the Indonesian Island of Sumatra from the Indian Ocean. As a benthic organism, it is likely that genetic flow in these species may be lower than for planktonic dinoflagellates due to the substrate attachment (Penna *et al.,* 2010) or land-derived nutrient requirements (Taylor *et al.,* 2008). Consequently, more notable geographical dispersion may be reflected in the phylogeny of benthic microorganisms (Penna *et al.,* 2012). In addition to that, ocean currents and ballast water, nowadays considered the most important mechanism of spread for microalgae (Hallegraef & Boch, 1991), may contribute to the dispersion and resulting genetic flow.

While Mediterranean strains have largely been proven to be PLTX-like compound producers (Ciminiello *et al.,* 2012), *O*. cf. *ovata* strains analyzed in this study did not demonstrate production of PLTX-like compounds. In agreement with this result, a strain of *O.* cf. *ovata* from Japanese waters, strain s0579, (same sequence as CAWD174 in Figure 14 - Sato *et al.,* 2011) that belonged to the same clade as the Reunion Island strains, was analyzed in Suzuki *et al.,*(2012) and no PLTX-like compounds were found. Toxin content and specific profiles may contribute to the species identification as an additional feature and may help to solve taxonomical problems. As reported in Penna *et al.,* (2010) and Parsons *et al.,* (2012), considering differences both in molecular and toxicological features, *O*. cf. *ovata* from the Mediterranean-Atlantic and Indo-Pacific clades may be classified as different species.

The other group of cells with a small morphotype constituted a genetically-homogeneous clade and did not cluster with any other sequence registered in Genbank. Morphologically they may fit among any of the small-oblong shaped cells described for the genus *Ostreopsis* with a possible

correspondance with *O. belizeanus* or *O. caribbeanus*, observed in the west Indean Ocean (Faust, 1999; Rhodes, 2011). At the same time, it may represent a new species not observed previously in the field. Further investigations are needed to taxonomically characterize this phylotype.

In our study, we evaluated toxicity in the stationary phase where a higher accumulation of toxin content in *O*. cf. *ovata* strains has been already observed (Vanucci *et al.,* 2012a; Vanucci *et al.,* 2012b; Scalco *et al.,* 2012; Pezzolesi *et al.,* 2012; Guerrini *et al.,* 2010, Carnicer *et al.,* submitted). As for culture conditions in our laboratory, irradiance was set according to standard procedures in our laboratory (20-40 µmol photons $m^{-2} \cdot s^{-1}$), and we understand these conditions could be a limiting factor since it is a much lower value than average values found in tropical regions (Badosa *et al*., 2013).

Our phylogenetic tree highlighted ten distinct clades and was supported by previous studies that clustered the same groups of strains together. The four species with an assigned sequence, *O*. cf. *ovata*, *O.* cf. *siamensis, O. labens* and *O. lenticularis* are clustered in the same way as reported in Penna *et al.,* 2010; Amzil *et al.,* 2012; Moreira *et al.,* 2012 and Kang *et al.,* 2013. Phylogenetic trees performed with new sequences from species not identified morphologically (Sato *et al.,* 2011; David *et al.,* 2013) are also in agreement with our results. The present study focusing on Reunion Island *Ostreospsis* strains highlights the occurrence of two morphotypes encompassing three phylotypes, one of which could constitute a new species. These results illustrate the need to reassess the controversial status of the taxonomy of the genus *Ostreopsis*. New sequences provided in this study help to define genotypes of previously described species, improve knowledge on their biogeography, and provides data for expanding the description of species within this genus. This study contributes important genetic, morphological, biogeographical and toxicological information to the description of the genus *Ostreopsis* that will clarify its problematic taxonomy.

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