Morphological, molecular and toxicological data on *Ostreopsis* cf. *siamensis* (Dinophyceae) from the Atlantic Iberian Peninsula

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Abstract

Ostreopsis siamensis, the type species of the genus *Ostreopsis*, was originally described from the Gulf of Thailand based only on morphological characters. Currently, this genus includes 11 accepted nominal species many of which are considered cryptic. Recently, phylogenetic and morphological studies have allowed the identification of well-supported clades, such as the one from temperate waters in Europe and Oceania tentatively named as *O*. cf. *siamensis*. That designation was based on morphological similarities with *O. siamensis*. However, a recent study on the distribution of *Ostreopsis* spp. in the Gulf of Thailand, where *O. siamensis* was originally described from, did not detect the European/Oceanian ribotype. Its absence suggests that the European/Oceanian ribotype does not include the type of *O. siamensis*. Morphological, molecular and biogeographic differences with other nominal species also preclude the application of any other name, which suggests that a new species should be assigned to this clade. In this study, we clarify the taxonomic status of the European/Oceanian ribotype known as *O*. cf. *siamensis*, based on morphological, molecular and toxicology data.

Keywords: Benthic Dinoflagellates, *Ostreopsis*, Phylogeny, Taxonomy, Toxicity

Introduction

The benthic dinoflagellate genus *Ostreopsis* Schmidt currently includes 11 species, many of which are known to be toxic, producing palytoxins analogues named ostreocins and ovatoxins (Fernández-Araujo et al., 2013).

This genus was described from the Gulf of Siam (currently Gulf of Thailand), Thailand, with the type species *Ostreopsis siamensis* Schmidt (1901). The original description highlighted the oyster-shaped flat body, the eccentric apex, the presence of a slit-shaped apical pore and porous plates. The original drawings of *O. siamensis* depicted a round-shaped cell in apical view (epitheca), a tear-shaped cell in antapical view (hypotheca), coarse porous plates and an undulated cell in side view. The reported cell size was large with a dorso-ventral (DV) axis of 90 µm. In 1981, Fukuyo applied this name to specimens from the Ryukyu Islands (Japan) with an undulated body in side view, and gave additional details, such as size range (DV of 60-100 µm; transdiameter of 45-90 µm). In the same work, Fukuyo added two new species to the genus, which were distinguished from *O. siamensis* based on the absence of cell undulation, size (*O. ovata)* and type of thecal pores (*O. lenticularis*). In the following years, six new species were described based on morphology (Norris et al., 1985; Quod, 1994; Faust & Morton, 1995; Faust, 1999).

However, the diagnostic characters limiting the different morphospecies often overlapped or were ambiguous, suggesting the occurrence of cryptic species or morphological plasticity (Parsons et al. 2012). Recently, two additional species (*O. fattorussoi* and *O. rhodesiae*) were described based on both morphological and molecular characterization (Accoroni et al., 2016; Verma et al., 2016a).

The use of molecular taxonomy has shown the existence of distinct ribotypes of *Ostreopsis*, with divergences supporting the description of species. However, establishing the link between the described species and the different clades has been hampered by the lack of biological type material (Pin et al. 2001, Penna et al. 2005, Sato et al., 2011). One such case is the well-defined ribotype *Ostreopsis* cf. *siamensis* first described from the Mediterranean Sea and morphologically similar to *O. siamensis* (Penna et al., 2005). Presently, the *O.* cf. *siamensis* clade includes strains from the Atlantic Iberian Peninsula, the Mediterranean Sea, New Zealand and Australia (David et al., 2013; Verma et al., 2016b), based on ribosomal sequence similarity.

A recent detailed phylogenetic study on the diversity of *Ostreopsis* in the Gulf of Thailand, including sites from the area where *O. siamensis* was originally described, allowed the identification of two major clades in the area, *O.* cf. *ovata* and *Ostreopsis* sp. 6, none of which coincided with the clade known as *O.* cf. *siamensis* (Tawong et al., 2014).

The present work aims at clarifying the taxonomic status of the ribotype known as *O.* cf. *siamensis*, based on previous studies and new morphological, molecular and toxicology data.
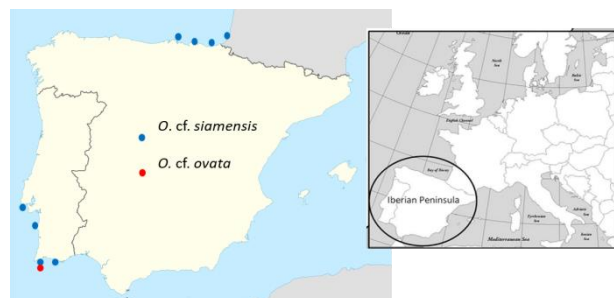
Materials and Methods

Fig. 1. Study and sampling area (Amorim et al. 2013, David et al. 2013; Laza-Martínez et al. 2011).

Study area and sampling

Epiphytic and planktonic samples were collected from the Atlantic coast of the Iberian Peninsula (Fig. 1). Samples were observed, and cells were isolated and cultured under controlled laboratory conditions (f/20; 19 ºC; 12L:D). Nineteen strains of *O.* cf. *siamensis* were successfully established and are presently kept at the algae culture collection of the University of Lisbon (ALISU).

Morphological analysis

Cultures and Lugol field-fixed samples were observed under the light and scanning electron microscopes, following the methods of David et al. (2013).

Phylogenetic analysis

Approximately 30 ml of exponentially growing cultures were harvested by centrifugation. DNA was extracted and purified using the GRS genomic DNA kit following the manufacturer's instructions (Grisp, Portugal). Amplification was carried out with primers ITSA and ITSB (Adachi et al., 1994) for the ITS-5.8S rDNA region according to Silva et al. (2015). Phylogeny was supported by Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbour Joining (NJ) methods. Uncorrected genetic pair-wise (p) distances were calculated from the ITS alignment using Mega7 software.

Toxin profile by Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS)

Cultures (10^5 – 10^6 cells) were centrifuged to separate cell pellets and culture media. Samples were kept frozen at -20ºC until analysis. Both pellets and media were extracted following Tartaglione et al. (2016) and all the extracts were analysed by LC-HRMS following Ciminiello et al. (2015).

Toxicity test with a mammalian cell line

Vero E6 cells were cultivated in 96-well culture plates, at 37ºC in DMEM medium supplemented with 10% foetal serum. The toxicity assay consisted in exposing cell cultures to dilutions of *Ostreopsis* cell extracts (0, 1/2, 1/4, 1/8) and respective culture medium. The cultures were incubated for 24 hrs at 37 ºC. A strain of toxic *O.* cf. *ovata* was used as positive control of toxicity and 100% viability correspond to non-treated Vero E6 cultures. The MTT viability assay was carried out and absorbances were recorded. Results reflect the number of viable cells present in each well.

Results and Discussion

Phylogeny

The alignment included 73 sequences, 25 from *O.* cf. *siamensis* of which 14 were from the present study. The sequences assigned to *O.* cf. *siamensis* grouped in a very homogeneous clade (Fig. 2), showing an intra-clade distance of 0.001 (Table 1).

Considering the genetic distance of *O.* cf. *siamensis* to other species within *Ostreopsis* (Table 1), the minimum genetic distance was 0.113 to *O. rhodesiae*. This value is well above 0.04, the value proposed by Litaker et al. (2007) as the minimum genetic distance allowing deli-neation of species in free-living dinoflagellates.
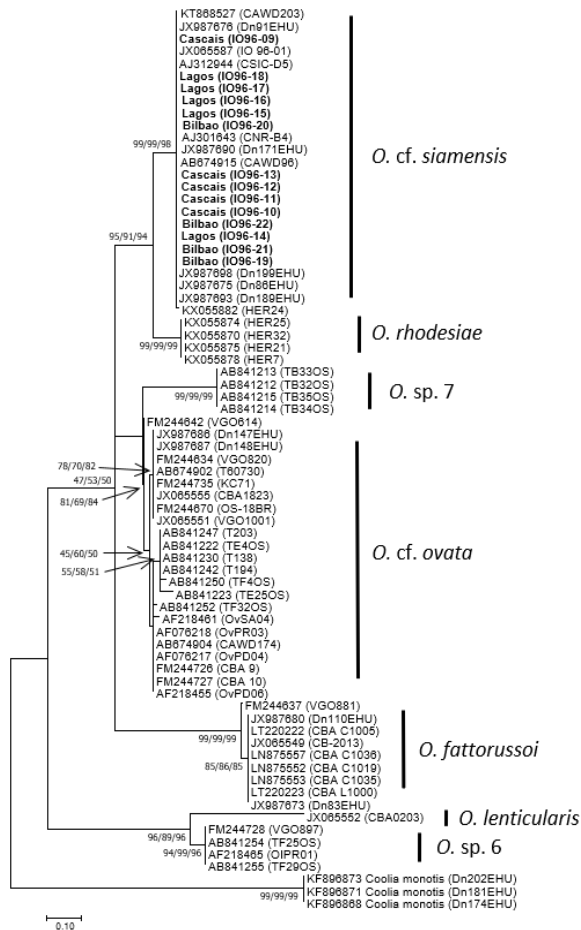

Fig. 2. ML phylogenetic tree of the genus Ostreopsis inferred from ITS-5.8S ribosomal gene sequences. The tree is rooted with Coolia monotis as outgroup. Numbers on the major nodes represent from the left to right NJ, MP, ML (1,000 pseudoreplicates).

Recent work on the diversity of the genus *Ostreopsis* in Thailand, identified two clades of *O.* cf. *ovata* and a clade identified as *Ostreopsis* sp. 6 in the Gulf of Thailand, where *O. siamensis* was described from, but did not detect strains belonging to the European/Oceanian ribotype of *O.* cf. *siamensis* (Tawong et al., 2014). In the absence of biological type material of *O. siamensis*, the latter study provides an insight into its possible molecular identity, namely it suggests that the type of *O. siamensis* belongs to one of the clades found by these authors, *Ostreopsis* sp. 6 (Fig. 2). This clade included *Ostreopsis* strains from the Gulf of Thailand, and from Japan and Malaysia. Tawong et al. (2014) gave further supporting morphological evidence that this could be considered the true *O.*

siamensis, namely the presence of cell undulation in lateral view, in conformity with the original description and drawings by Schmidt (1901).

Table 1. Uncorrected genetic ρ-values (net average genetic distances) between *Ostreopsis* sequences included in the phylogenetic analyses. In diagonal are within-clade distances.

		I	II	III	IV	V	VI	VII
I	*Ostreopsis cf. ovata*	0.023						
II	*Ostreopsis cf. siamensis*	0.226	0.001					
III	*Ostreopsis rhodesiae*	0.231	0.113	0				
IV	*Ostreopsis fattorussoi*	0.321	0.391	0.359	0.004			
V	*Ostreopsis sp. 7*	0.178	0.338	0.302	0.341	0		
VI	*Ostreopsis lenticularis*	0.786	0.547	0.516	0.537	0.694	n/a	
VII	*Ostreopsis sp. 6*	0.467	0.443	0.423	0.441	0.443	0.286	0
VIII	*Coolia monotis*	0.549	0.586	0.647	0.785	0.587	0.735	0.689

The absence of the *O.* cf. *siamensis* clade from the area where *O. siamensis* was described, and the well-defined ribotype *O.* cf. *siamensis,* with 0.113 as the minimum value of divergence with its closest clade, means there is support from phylogeography to its consideration as a separate species.

Morphological description

Cells are markedly antero-posterior compressed, tear-shaped to almost round. Cells with no undulation in side view. Dorsoventral diameter in cultures and field: 26-83 µm (54.3 ± 13.8 µm); Width: 13-71 µm (37.1 ± 9.8 µm). Cells in culture show morphological variability even within clonal strains. Figure 3 illustrates the observed variability: elongated vs round hypotheca (Fig. 3a, b), different size of the 7″ plate (Fig. 3d, e), diversity in cell size (Fig. 3c) and variable apical pore (Fig. 3f, g). Thecal plates are smooth, covered with randomly distributed pores sometimes with two distinct sizes as previously noticed (David et al., 2013). Plate formula Po, 3′, 7″, 5‴, 2⁗ and 1p.

Distribution

Ostreopsis cf. *siamensis* is common in the Atlantic coast of Iberia (Amorim et al., 2010, 2013; Laza-Martínez et al., 2011; David et al., 2013). Based on molecular data, this species is also present in the Mediterranean Sea and in the South West Pacific (New Zealand and Australia).

Toxicity

The presence of all the palytoxin analogues so far known (>20) was investigated by LC-HRMS but none of the known congeners was detected. Some potentially new palytoxin congeners, based on their characteristic ionization behaviour, could be present. Further investigation is necessary for their characterization.

The Vero cells assay indicated that all analysed strains of *O.* cf. *siamensis* had toxicity. Cytotoxicity levels decreased from the undiluted samples to the 1/8, from 83-94% of toxicity to 0-19%. The positive control always showed toxicity over 94%.
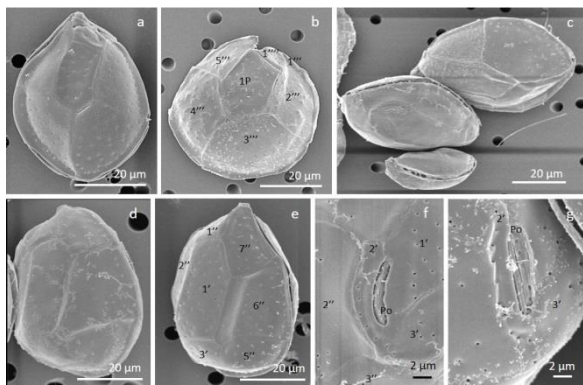

Fig. 3 – SEM micrographs of *O.* cf. *siamensis* showing variability of cultured strains. a, b - hypotheca, c - group of cells, d, e - epitheca, f, g - Apical pore plate and plate 2′.

Conclusions

Ostreopsis cf. *siamensis* is a well-defined ribotype that is not present in the area where *O. siamensis* was originally described. It is present along the Atlantic coast of Iberia, the Mediterranean Sea and in a few locations in the south Pacific. Given the genetic distinctiveness and the phylogeo-graphic differences with its morphologically similar species, we propose that the referred ribotype *O.* cf. *siamensis* should be considered a species different from *O. siamensis* Schmidt and other nominal species. A detailed comparative morphological analysis should show to what extent it is morphologically cryptic in respect to other similar species.

Acknowledgements

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