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Phylogenetic relationships of *Cochlodinium polykrikoides* Margalef (Gymnodiniales, Dinophyceae) from the Mediterranean Sea and the implications of its global biogeography

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ABSTRACT

Although the diversity of dinoflagellates has been intensively studied in several locations in the Mediterranean Sea since the 1950s, it is only during the last two decades that the morphotype of the toxic unarmoured dinoflagellate *Cochlodinium polykrikoides* Margalef has been detected, coinciding with its apparent worldwide expansion in marine coastal waters. In this study, vegetative cells of *C. polykrikoides* morphotype from the Catalan coast (NW Mediterranean Sea) were detected and isolated, and the DNA from collected cells was sequenced. While in the Mediterranean Sea, detections are scarce and *C. polykrikoides* is consistently present at low concentrations, we reported exceptional blooms of this species, in which the maximum abundance reached 2×10^4 cells L^{-1}. Partial LSU rDNA region sequences showed that most *C. polykrikoides* populations from the Catalan coast formed a new differentiated ribotype, but others were included within the 'Philippines' ribotype, demonstrating their coexistence in the Mediterranean Sea. Thus, the current biogeographic nomenclature of the ribotypes is likely to be invalid with respect to the available information from populations comprising the 'Philippines' ribotype. The phylogeny suggests the existence of cryptic species that should be evaluated for species-level status. Accordingly, the ribotype determination must be carefully evaluated for all detections and bloom events, since accurate characterization of the morphology, ecophysiology and distribution of the ribotypes are not well resolved.

1. Introduction

In phytoplankton, there are several examples in which high genotypic and phenotypic variability has been evidenced within what is considered to be the same species. Consequently, biogeographic information for phytoplankton is thus far limited since it requires accurately defined species. Phylogenetic studies on diverse microalgae have revealed that several traditional morphospecies are genetically distinct at a geographic scale, e.g., *Emiliania huxleyi* (Hagino et al., 2011), while cryptic species coexist in some locations, as shown for the *Pseudo-nitzschia delicatissima* complex (Quijano-Scheggia et al., 2009). Proper identification of the target species, including those that are toxic or noxious, is therefore often hindered. However, their detection is crucial due to the worldwide threat posed by harmful algae to human health, aquaculture, wild life, and ecosystem functioning. Furthermore, the identification of some species is extremely difficult when based only on fixed samples, which highlights the importance of using molecular methods to identify and quantify toxic and noxious organisms.

Cochlodinium polykrikoides Margalef is a bloom- and chain-forming unarmoured dinoflagellate responsible for high mortalities of wild and farmed fish (Kim, 1998). The first blooms of *C. polykrikoides* were reported prior to 1990 in Southeast Asia (Kim, 1998; Yuki and Yoshimatsu, 1989) and along the east coast of North America (Ho and Zubkoff, 1979; Tomas and Smayda, 2008). Since then, blooms of this species have expanded to the East China Sea, the Philippines, Malaysia, the west coast of North America, Costa Rica, and, in the last decade, in south-western Asia and Europe (Kudela and Gobler (2012) and references therein). In the Mediterranean Sea, *C. polykrikoides* was first detected in the late 1990s, initially in eastern Sardinia, the Gulf of Naples (Italy) (Sannio et al., 1997; Siano et al., 2002; Zingone et al., 2006), and, later, in the Adriatic Sea (Saracino and Rubino, 2006). Resting cysts of this species in sediments from the Mediterranean have been reported in the Adriatic Sea (Saracino and Rubino, 2006), the Ionian Sea (Rubino et al., 2010), and recently by Satta et al. (2013) in Alfacs Bay (Catalan coast, NW Mediterranean Sea).

The detection of *C. polykrikoides* resting cysts in Catalan waters and the observation of fixed chains of unarmoured dinoflagellates

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in samplings from the region's monitoring programme carried out in the area, raised our suspicions that *C. polykrikoides* was established along the Catalan coast. Confirmation of these suspicions, as described herein, was based on the detection of vegetative cells of *C. polykrikoides*, the first such report involving the western Mediterranean. Misidentification of this species was avoided by using single-cell PCR to sequence the partial large subunit (LSU) rDNA region from the isolated vegetative cells. This approach resulted in the first elucidation of the phylogenetic relationship between the organism isolated from the Mediterranean Sea and *C. polykrikoides* populations from other geographic regions. It also allowed an analysis of the biogeographic implications for this species.

2. Materials and methods

2.1. Sampling and isolation

During 2011 and 2012, fresh samples were obtained from coastal stations (9 harbours and 6 beaches along the Catalan coast, NW Mediterranean) (Fig. 1), with monthly to weekly samplings throughout the year. From each sample, one sub-sample was fixed with Lugol's iodine and 50 ml were allowed to settle for 24 h in a settling chamber. *C. polykrikoides* cell abundances were determined based on observations at a 200× magnification of an appropriate area, made using a Leica-Leitz DM-Il inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). The remaining live sample was concentrated through a 10-μm mesh, allowed to settle in a settling chamber, and then observed under a Leica-Leitz DM-Il inverted microscope. Since all efforts to obtain cultures from the isolated specimens were unsuccessful, microscopy observations were performed directly on living cells from the concentrated samples. Target organisms were filmed and photographed with an Alpha NEX5 camera (Sony) adapted to the microscope and subsequently isolated with a micropipette, washed in several drops of filtered seawater, and placed in a 200-μl PCR tube for further analysis as described below.

2.2. Extraction, amplification, and sequencing

For some samples, DNA was extracted from the cells following the method of Kai et al. (2006). Briefly, 5 μl of lysis buffer (0.005% SDS with 400 ng proteinase K μl^{-1}) was added to each 200-μl tube after which the tubes were frozen at −80 °C for at least 10 min. The samples were then incubated first at 60 °C for 30 min, then at 95 °C for 10 min to inactivate the proteinase K, and finally stored at −80 °C until processed. For other samples, the cells were transferred directly to the PCR tube, adding the minimum volume of seawater, followed by several rounds of freezing/thawing. The PCR mixture contained 5 μl of 10× buffer (Qiagen), 1.25 U of Taq DNA polymerase (Qiagen), 0.2 mM of each dNTP, and 0.8 μM of the primers D1R and D2C (Scholin et al., 1994). The PCR conditions were as follows: an initial denaturation for 5 min at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, followed by a final extension step for 7 min at 72 °C. Ten microliter of the PCR products were electrophoresed for 20–30 min at 120 V in a 1.2% agarose gel and visualized under UV illumination. The remainder was frozen at −20 °C until used for sequencing. Purification and sequencing were carried out by an external service (Genoscreen, France). Sequencing was done using the D1R primer and a 3730XL DNA sequencer.

2.3. Phylogenetic analyses

Sequences obtained in this study were deposited in Genbank and aligned with those obtained from GenBank (Table 1) using the MAFFT v.6 program (Katoh et al., 2002) under FFT-NS-i (slow; iterative refinement method) and manually checked with BioEdit v. 7.0.5 (Hall, 1999), obtaining a final alignment of the D1–D2 region of about 760 positions. Phylogenetic relationships were determined using the maximum-likelihood (ML) and Bayesian inference methods. For the ML method, the GTRGAMMA evolution model was used on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with the RaxML software. The Bayesian inference was performed with MrBayes v.3.2 (Ronquist et al., 2012), run with a GTR model with rates set to gamma. Each analysis was performed using four Markov chains (MCMC), with one million cycles for each chain. The consensus tree was created from post-burn-in trees and the Bayesian posterior probabilities (BPP) of each clade were examined.

3. Results

3.1. Detection

C. polykrikoides was detected in three harbours (Fig. 1) during 2011 and 2012 but never in samples from beaches. The cell abundances were low ($<10^4$ cells L^{-1}) and the detections were restricted to the summer months (from June to September), with water temperatures of 23.4–24.8 °C and salinities of 31.4–38.2 (Table 2). However, in Arenys Harbour, *C. polykrikoides* was detected in 2011 over a period of 2 months, reaching a maximum abundance of 2×10^4 cells L^{-1} in July 2011, but it was not detected again in that location in 2012.

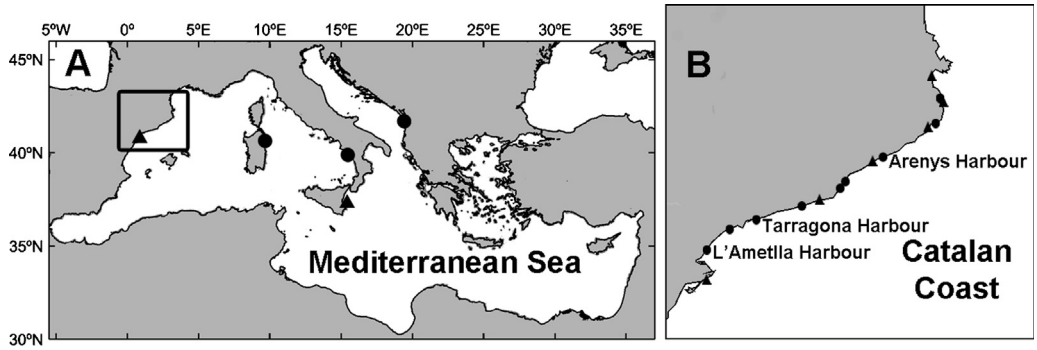

Fig. 1. Sampling locations. (A) Sites from the Mediterranean Sea where *Cochlodinium polykrikoides* has been previously reported. Dots indicate sites of vegetative cell detections and triangles those of resting cysts. (B) Locations sampled during this study. Dots indicate harbours, and triangles beaches. Only the locations where *C. polykrikoides* was detected are labelled.