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High genetic variability of *Alexandrium catenella* directly detected in environmental samples from the Southern Austral Ecosystem of Chile

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ABSTRACT

Chilean waters are often affected by *Alexandrium catenella*, one of the leading organisms behind Harmful Algae Blooms (HABs). Genetic variability for this species are commonly carried out from cultured samples, approach that may not accurately quantify genetic variability of this organism in the water column. In this study, genetic variability of *A. catenella* was determined by sequencing the rDNA region, in water samples from the Canal Puyuhuapi (South Austral Ecosystem of Chile). *A. catenella* was detected in 8,8% of samples analysed. All sequences obtained were *A. catenella* (Tamara complex group I), with three highly frequent haplotypes (34%), and twenty new haplotypes. These haplotypes increase the genetic variability from 2.8% to 3.14% in this area. Through this new method, genetic determination of *A. catenella* can accurately be monitored and ecological studies of this species can be implemented.

1. Introduction

Dinoflagellates of the genus *Alexandrium* are the greatest cause of the harmful algal bloom (HABs) syndrome known as Paralytic Shellfish Poisoning (PSP). In recent decades, HABs caused by this genus have increased in frequency, severity, and geographic distribution (Wang et al., 2008), affecting marine ecosystems and the safety of seafood (Sellner et al., 2003; Heisler et al., 2008). The genus *Alexandrium* has been separated into two major species complexes based on morphological similarity and ribosomal sequence likeness: the *minutum* group (*A. lusitanicum*, *A. angustitabulatum*, *A. andersonii* and *A. minutum*) and the *Alexandrium tamarense* complex (originally grouped as "*Alexandrium tamarense*", "*A. catenella*" and "*A. fundyense*"), (Scholin et al., 1995; Wang et al., 2008). Recently based on morphogenetic studies, a formal taxonomic revision of the *A. tamarense* complex species has been proposed, identifying five genetic clades as different species: *A. fundyense* (Group I; former *A. catenella*); *A. mediterraneum* (Group II), *A. tamarense* (group III); *A. pacificum* (Group IV); A. *australiense* (Group V) (Lilly et al., 2007; Miranda et al., 2012; John et al., 2014; Wang et al., 2014). However, a recent report established that *A. fundyense* and *A. catenella* are conspecific, and *A. fundyense* should be named to as *A. catenella* (Prud'homme van Reine, 2017). Numerous strains of the *A. tamarense* complex have caused severe PSP outbreaks in coastal regions around the world, with *A. catenella* and *A. tamarense* being the species most frequently associated with such events (Hallegraef, 1993; Kamikawa et al., 2007). Chile has been hit repeatedly by HABs linked to *A. catenella* since 1972 in the Magallanes Region (Guzmán and Campodónico, 1975), and currently detected from the Aysén Region to Chiloé Island (Mardones et al., 2010), causing a variety of negative impacts that range from economic losses to human deaths. This discourages bivalve shellfish aquaculture and fisheries activities in affected areas (Uribe et al., 2010). This situation, has promoted the establishment of a monitoring program by government authority, based on specific qualitative/quantitative monitoring of *A. catenella* in both the water column and in sediments. These routine analyses are based on microscopic observations of taxonomic features, including differences in the shape of plates, length to width ratios and presence or absence of a ventral pore (Balech, 1985; John et al., 2014). However, many authors concluded that this approach is inconclusive for species identification, among other reasons, due to the presence of cells with intermediate morphologies, providing erroneous information regarding the geographical distribution, ecology and toxicity of this genus (Lilly, 2003; Gayoso and Fulco, 2006; John et al., 2014; Wang et al., 2014). It is therefore necessary to complement the taxonomic approach with molecular tools to ensure correct species identification (John et al., 2003; Lilly et al., 2007; Touzet et al., 2007; Touzet et al., 2008).

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Combinations of both molecular tools and bioinformatics analyses, mainly based on DNA ribosomal sequences, have allowed inter- and intra-species identification, as well as determination of genetic variability of dinoflagellates (Godhe et al., 2001; John et al., 2003; Galluzzi et al., 2005; Lilly et al., 2007; Touzet et al., 2007; Touzet et al., 2008; Aguilera et al., 2011; Richlen et al., 2012; John et al., 2014). Genetic variability drives evolution and is a characteristic of all species including harmful algae and is critical for their survival (Rynearson and Ambrust, 2005). In fact, many reports have shown genetic variability in environmental samples of *A. minutum*, *A. tamarense*, *A. catenella*, and *A. fundyense* (Chow et al., 2004; Penna et al., 2005; Alpermann et al., 2010; Aguilera et al., 2011; Erdner et al., 2011; Richlen et al., 2012). Generally, these studies involve the isolation of organisms from the environment and the establishment of clonal cultures, however limits of *in vitro* culture and loss of some strains while attempting to culture could in fact underestimate the genetic variability of this species and lead to erroneous conclusions about this species in its natural condition (Burkholder and Glibert, 2006). For phylogenetic analyses to be of utility a genetic marker of sufficient variability should be used. Recent work by Wang et al. (2014) have suggested a revised taxonomy for the *A. tamarense* complex based upon the ITS rDNA region of the genome, as other ribosomal markers show poor resolving power for separating genetically distinct lineages.

Therefore, the aim of the present work was to develop an effective molecular method to detect *A. catenella* in natural conditions and avoid culture methods. Molecular data was used to determine the genetic variability of this population, and was then compared with sequences reported at different geographical sites from the Southern Austral Ecosystem of Chile (SAE). This assay provides an alternative method for accurately monitoring and performing ecological studies of this dinoflagellate throughout the world's oceans.

2. Methods and materials

2.1. Study area and sample collection

Samples were obtained from the water column of the Puyuhuapi Channel (from 44°19′72°33′W to the North, to 44°57′S, 73°21′W to the South), at 24 water column stations between November 2010 and September 2012 (Fig. 1). Water samples (0,5 L) were obtained with Niskin bottles at depths of 0, 10, 25 and 50 m. The water was prefiltered at 100 μm, and then filtered through a cellulose ester filter with a diameter of 47 mm and pore size of 0.22 μm with a pressure of less than 100 mm Hg. Filters were stored at − 80 °C until processing.

2.2. Total DNA extraction and determination of concentration and quality

Total DNA extraction from filters was performed with 0.25 mL of extraction buffer (Tris-HCl 20 mM pH 5.2, EDTA 50 mM, SDS at 10% (*W/V*), and Proteinase K (10 mg mL^{-1}), followed by an incubation at 50 °C for 10 min, and a subsequent freeze for 15 min. This procedure was repeated three times. The lysis mixture was extracted with saturated phenol (neutralized with Tris-HCl 0.5 M, pH 7.8 to 8.5), and chloroform-isoamyl alcohol (24:1). Then, the nucleic acids were precipitated with 2,5 volumes of ethanol 95% (vol/vol) at − 20 °C in presence of 0.1 volumes of sodium acetate 3 M (pH 5.2). The mixture was incubated at − 20 °C overnight, and centrifuged at 21,000*g* for 20 min. The pellet was washed twice with cold ethanol 70% and air dried for 10 to 15 min. The pellet was resuspended in 100 μL sterile water and stored at − 80 °C. DNA concentration and purity was estimated by spectrophotometry in a Nanodrop 2000. The quality of the extracted DNA was evaluated by electrophoresis (100 V, 45 min) in a 1% agarose gel, stained with ethidium bromide (5 mg mL^{-1}).

2.3. Primer design, PCR amplification and specificity test

To design primers, sequences for *Alexandrium catenella* ITS1-5.8S-ITS2 ribosomal region (~520 base pairs) obtained in both COPAS Sur Austral (personal database) and NCBI database (using "*A. catenella* AND Chile" as search), were analysed. These sequences combined with ITS sequences originating from representative genetic clades of *Alexandrium tamarense* complex, were aligned with Vector NTI Advance ® software version 10.3.0 (Invitrogen TM), and species-specific primers were designed in highly conserved regions for the species of study, *Alexandrium catenella*. All samples were analysed by polymerase chain reaction (PCR), with Taq 1X PCR buffer (Promega), 1 U GoTaq® Flexi DNA polymerase (Promega), 3.0 mM $MgCl_2$, 0.4 mM of each dNTPs (Promega), and 20 pmoles of each forward and reverse primer in a 25 μL reaction volume. To determine the minimum amount of DNA to carry out PCR reactions, DNA isolated from monoclonal culture cells of *A. catenella* strain PFB 37 (HM641243), was amplified. The optimum DNA quantity amplified was 5 ng, with a range of 15–0.16 ng (data not shown). The PCR program was: a preliminary denaturation step at 95 °C for 5 min; 5 cycles of pre-amplification of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s; then, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; and a final extension of 72 °C for 3 min. All the reactions were performed in a Thermocycler T-personal Biometra®. The specificity of the designed primers were evaluated using genomic DNA from vegetative cells or cysts of *Alexandrium minutum*, in presence and absence of gDNA from *A. catenella* strain PFB 37 (HM641243). All PCR products were assessed by electrophoresis (100 V, 45 min.) in agarose gel at 1%, stained with ethidium bromide (5 mg mL^{-1}).

2.4. Cloning and purification of plasmids

The PCR product corresponding to the expected amplification size was cut from the agarose gel, and purified using the Gel extraction kit E.Z.N.A. (Omega Biotek) according to the manufacturer's instructions. Purified DNA was ligated into the pGEMT-Easy vector and transformed to competent *Escherichia coli* JM109 cells using the thermal shock method. Positive white colonies were selected from X-gal/amp agar plates and grown in LB/amp liquid medium. Plasmids were purified using the Plasmid Miniprep kit II E.Z.N.A. (Omega Biotek), following the manufacturer's instructions. All positives clones were checked by PCR amplification.

2.5. Sequencing and phylogenetic analysis

Sequencing of the purified plasmids containing the cloned fragment of ITS1-5.8S-ITS2 from *A. catenella* ribosomal DNA was performed by MACROGEN (Korea) using BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA). To verify that the sequenced clones corresponded to the study species, preliminary analysis of the sequences obtained was performed using the BLAST program. In order to compare sequences of ITS1-5.8S-ITS2 rDNA, aligned haplotype diversity (Hd) was calculated using DNA Sequence Polymorphism software version 5.10.01 (Librado and Rozas, 2009). For phylogenetic analyses, representative ITS1-5.8S-ITS2 rDNA sequences of *A. catenella* from the Southern Austral Ecosystem of Chile and ITS sequences originating from representative genetic clades of the Tamarense complex available in GenBank were used (Additional Table 1), and defined as CM: this study; AA: sequences described in Aguilera et al., 2011; and VV: sequences described in Varela et al., 2012. The ITS1-5.8S-ITS2 rDNA sequence from *A. minutum* strain AM 18S was used as the outgroup. Sequences were aligned and manually edited using MEGA 6 Software (Tamura et al., 2013). jModeltest version 2.1 (Darriba et al., 2012) was used to determine the best substitution model and associated parameters for the phylogenetic analysis. The model selected for ITS analysis based on the Akaike Information Criterion (AICc) was HKY + G with base frequencies of A: 0.247, C: 0.151, G: 0.244, T: 0.358;