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Molecular phylogeny and toxin profiles of *Alexandrium tamarense* (Lebour) Balech (Dinophyceae) from the west coast of Greenland

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ABSTRACT

Detection of paralytic shellfish poisoning (PSP) toxins in scallops from the west coast of Greenland exceeding the 800 μg toxin/kg shellfish limit led to an investigation with the aim of finding the responsible organism(s). Three strains of *Alexandrium* Halim were established from single cell isolations. Morphological identification of the strains and determination of their position within the genus by LSU rDNA sequences was carried out. Light microscopy revealed that the three strains was of the *Alexandrium tamarense* morphotype, and bayesian and neighbor-joining analyses of the LSU rDNA sequences placed them within Group I of the *A. tamarense* species complex. The toxicity and toxin profiles of the strains were measured by liquid chromatography fluorescence detection (LC-FD) and their identity was confirmed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The three strains all turned out to be toxic and all produced large proportions (>60% total mol) of gonyautoxins 1 and 4 (GTX1/GTX4). This is the first record of saxitoxin producers from western Greenland. The toxin profiles were atypical for *A. tamarense* in their absence of N-sulfocarbanoyl C1/C2 or B1/B2 toxins. Rather the high molar percentage of GTX1/GTX4, the lesser amounts of only carbamoyl toxins and the absence of decarbamoyl derivatives are more characteristic features of *A. minutum* strains. This may indicate that the genetically determined toxin profiles in *Alexandrium* species are more complex than previously appreciated.

1. Introduction

The marine dinoflagellate *Alexandrium tamarense* (Lebour) Balech occurs worldwide, but with a tendency for biogeographical bias toward temperate coastal waters (Steidinger and Tangen, 1997). This dinoflagellate is notorious as one of the most well known species to produce the tetrahydropurine neurotoxins that cause paralytic shellfish poisoning (PSP). Saxitoxin (STX) and more than two dozen naturally occurring derivatives (collectively PSP toxins) are potent neurotoxins that block the sodium-channels in cell membranes. The PSP toxin syndrome in humans is characterized by primarily neurological symptoms – tingling and numbness in the extremities, with paralysis leading to death by respiratory arrest in severe cases (Kao and Walker, 1982; Clark et al., 1999). Most PSP toxicity events are caused by ingestion of contaminated shellfish, primarily suspension-feeding bivalve molluscs, which accumulate the dinoflagellate toxins in their flesh (Bricelj and Shumway, 1998).

A. tamarense is also capable of forming Harmful Algal Blooms (HABs), in some cases responsible for marine faunal mortalities, including fish kills (Cembella et al., 2002). In recent years *A. tamarense* has received heightened interest due to the fact that HABs of this species (as well as other toxic microalgae) seem to be increasing worldwide (Hallegraeff, 1993).

The taxonomic status of *Alexandrium* at both the genus and species level has long been a matter of debate, but recent controversies regarding *A. tamarense sensu* Balech (1995) have centered on the description as a valid species. Scholin et al. (1994) sequenced the large subunit (LSU) rDNA gene of several strains of *A. tamarense*, *A. catenella* and *A. fundyense*, as well as other species of *Alexandrium*, and found the strains to comprise five clades ("ribotypes"), of which two held more than one species. This shed further light on earlier analyses based on phenotypes of enzyme electrophoretic profiles (Cembella et al., 1988) and the view of *A. tamarense*, *A. catenella* and *A. fundyense* as a species complex rather than three morphologically distinct species. Further molecular investigations (Sebastian et al., 2005; Lilly et al., 2007) have confirmed the existence of five genetically distinct clades, two of which hold all three different morphotypes. Only two of the clades contain strains that have been confirmed to produce PSP toxins

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(Lilly et al., 2007), and both are polyphyletic with regard to morphospecies. The most recent taxonomic and phylogenetic view of *Alexandrium* (Anderson et al., 2012) suggests that these clades indeed represent cryptic species.

The risk of blooms of *A. tamarense* and the associated PSP toxicity is of particular importance in areas where a high proportion of the economy is based on export and/or local consumption of seafood. This applies to Greenland where the scallop industry has existed for more than two decades. In the 1980s stock assessments were carried out in many places along the west coast, and scallop beds were found sporadically with only a few being commercially viable. In the areas where the populations were exploitable, based on the assessments and knowledge of growth rates and recruitment, TAC (total allowable catch) quotas were advised to be set at 10% of the stock and minimum landing size of 65 mm. Today scallops are dredged at more than 10 locations along the west coast, and the catches have increased from 410 tons in 1984–2240 tons in 2002 (Anonymous, 2004; Garcia, 2006). In 2002 the export value of scallops from Greenland was approx. €5.5 million (Anonymous, 2003). Recently a decrease in fleet size has resulted in lower catches and export (H. Siegstad, personal communication), but with proper management based on new stock assessments and conservative TAC quotas the scallop industry could be viable (Garcia, 2006).

Following the detection in 2003 of PSP toxicity levels in excess of the EU regulatory limit of 800 μg saxitoxin equivalents (STX eq) kg^{-1} shellfish flesh, harvest of scallops in the Attu area was banned (B.R. Thorbjørnsen, personal communication). The Attu area (67°50′N–68°10′N, 53°00′W–54°00′W) covers approximately 1500 km^2 on the west coast of Greenland (Fig. 1) and 132 tons of scallops were caught in the area in 2002 (Anonymous, 2004). This amounted to 6% of total catches on the Greenland west coast. The detection of PSP toxicity was by the AOAC mouse bioassay, but the organism(s) responsible for the toxicity in scallops was not identified. In 2005, plankton samples were taken in the area with the aim of identifying the organism(s) and additional samples were collected further south in Maniitsoq (Fig. 1). A number of putative *Alexandrium* cells were isolated into culture for further study at University of Copenhagen. The *Alexandrium* clones were examined morphologically, genetically (i.e. LSU rDNA sequencing) and with respect to PSP toxin content and composition.

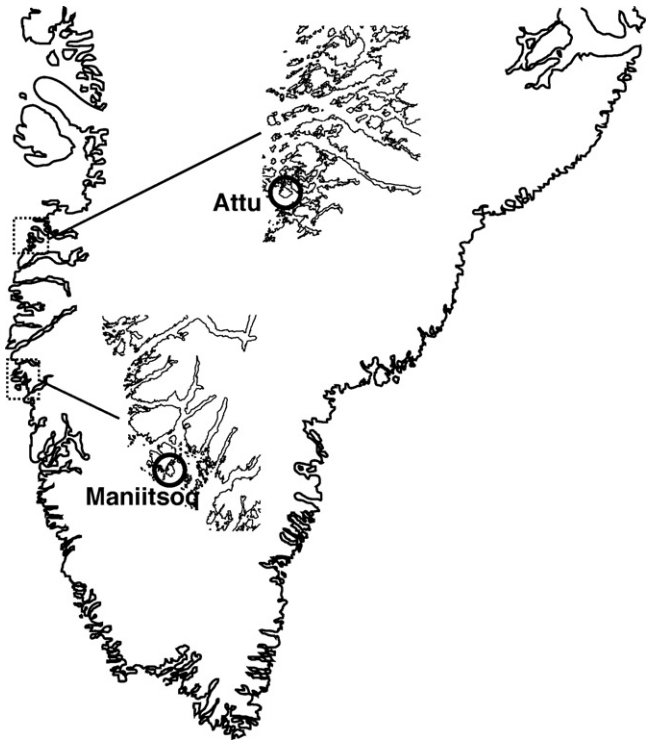

Fig. 1. Map of Greenland below 72°N. Sampling areas on the west coast are indicated by dashed squares. Sampling sites in Attu and Maniitsoq are shown by circles on the detailed maps.

Here we present the first gene sequences of the *A. tamarense* species complex from above the Arctic Circle, allowing elucidation of the phylogenetic position of the *Alexandrium* isolates from the west coast of Greenland. Furthermore, to our knowledge we have provided the first PSP toxin profiles of *Alexandrium* isolates from the western Arctic, establishing unique features of the toxin composition and variations among conspecific strains from Greenland. We conclude that *A. tamarense* populations from this region are toxigenic and that this species is the most likely candidate to account for the PSP toxicity recorded in the scallops.

2. Materials and methods

2.1. Isolation and cultivation

Plankton samples were collected with a phytoplankton net (mesh size 20 μm) off the coast of Attu (vertical tow) and at the entrance to Maniitsoq Harbor (surface tow), both on the west coast of Greenland, in August 2005 (Fig. 1, Table 1). Single cells were isolated by capillary pipettes and placed separately into wells of a 96-well tissue culture plate containing drops of T30 growth medium (Larsen and Moestrup, 1994). After a few cell divisions, the contents of each well were transferred to 40-ml culture flasks. The cultures were initially incubated at 4 °C but due to a very low cell division rate they were transferred to 10 °C and maintained on a 14:10 h light:dark cycle at a photon flux density of ca. 30 μmol m^{-2} s^{-1}. Despite numerous isolation attempts, only three cultures were established (K-0973, K-0974, and K-0975), now available at the Scandinavian Culture Center for Algae and Protozoa (SCCAP) in Copenhagen. Three other cultures reached a few cells (A1, D2, and E1); these were isolated for single-cell PCR and determination of LSU rDNA.

2.2. Light microscopy

Light microscopy of whole cells was performed with a Zeiss Axioplan fitted with a Zeiss Axiocam HR digital camera (Zeiss, Oberkochen, Germany). Thecal plate tabulations were assigned according to the Kofoid (1909) notation system, from unstained specimens prepared by amphiesmal plate squashes.

2.3. DNA analyses

2.3.1. LSU rDNA amplification

Five to six cells were isolated by capillary pipette from each culture, washed in fresh medium and transferred to Eppendorf tubes. A preheating step was performed to lyse the cells by adding 1 μl of Taq buffer (167.5 mM Tris–HCl, pH 8.5, 5 mM $(NH_4)_2SO_4$ and 25 mM β-mercaptoethanol) and 7 μl of double-distilled H_2O to each tube, and the tubes were then heated to 94 °C for 10 min.

Table 1
Location, coordinates and dates of collection. The strains/isolates K-0973, K-0974 and K-0975 are available from Scandinavian Culture Collection for Algae and Protozoa.

Location	Coordinates	Date	Strain/Isolate code
Attu, Gl	67°56′N, 53°35′W	16.08.2005	K-0973, K-0974, E1
Maniitsoq, Gl	65°25′N, 52°54′W	20.08.2005	K-0975, A1
Maniitsoq, Gl	–	21.08.2005	D2