Elsevier Editorial System(tm) for Harmful Algae
Manuscript Draft

Manuscript Number: HARALG-D-12-00041R1

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

Article Type: Original Research Article

Keywords: Alexandrium tamarense
Greenland
LC-FD
LC-MS/MS
LSU rDNA
PSP toxins
Toxic dinoflagellates

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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 A. tamarense morphotype, and bayesian and neighbour-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.

Dear Dr Sandra Shumway, editor of Harmful Algae

We have just submitted electronically a manuscript addressing for the first time toxic isolates of the *Alexandrium tamarense* morphotype from the west coast of Greenland. With this study we have filled one of the gaps in our understanding of PSP toxin producers in Greenlandic waters. Our study was initiated when in 2003 scallops from the Attu area had PSP toxin values exceeding the 80-µg toxin/100 g shellfish. Harvesting has been was banned ever since. Øjvind Moestrup went sampling in August 2005. He brought back the water samples that formed the basis of this study.

In brief the study is based on 6 isolates of the *A. tamarense* morphotype from the area of Attu and Maniitsoq. For reasons of identification and phylogenetic inference the nuclear-encoded LSU rRNA was determined from all of these. However, since only three of the isolates grew well in under the culture conditions provided we were only able to examine the toxin profiles for these. For this we used both LC-FD and LC-MS/MS. We also performed a more traditional identification of the isolates by careful examination of the thecal plate arrangement under the light microscope.

In addition to presenting novel toxin profiles for three isolates of *A. tamarense* these revealed to be atypical for this species. In fact their toxin profile is more similar to that of *A. minutum*. This is interesting since toxin profiles characteristic for species of *Alexandrium* is thought to be genetically determined. Additional studies will have to explore this finding in more detail.

Once the manuscript has been accepted we will submit the LSU rDNA sequences determined here to Genbank and include the accession numbers in the text.

With this manuscript we see no ethic or conflict of interest.

The work has not been published or submitted previously.

All authors approved the final version of the manuscript.

Brief description of contribution by authors

Claus Baggesen: Was the main responsible person for the study. He kept the strains of Alexandrium, took the micrographs, did the molecular work with Niels Daugbjerg, the toxic analyses with Bernd Krock and Allan Cembella. Claus wrote the first manuscript draft.

Øvind Moestrup: collected the water samples in Greenland and made the clonal cultures, which formed the basis of this study. Assisted in preparing the final version of the manuscript.

Niels Daugbjerg: supervised the molecular work and did the phylogenetic analyses. Assisted in preparing the final version of the manuscript. ND was responsible for getting the manuscript ready to be submitted.

Bernd Krock: Responsible for the toxic analyses and assisted.

Allan Cembella: Responsible for the toxic analyses and assisted in preparing the final version of the manuscript.

Sine Madsen: Responsible for organizing the collection of water samples in west Greenland.

Among possible reviewers are:

Dr Santiago Fraga (santi.fraga@vi.ieo.es)

Dr Donald Anderson (danderson@whoi.edu)

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We look forward to hear from you.

Best wishes,

Niels Daugbjerg.

***Detailed Response to Reviewers**

Response to reviewers.

The ms need very little revising.

1. Fig 4 has been updated according to the editor
2. Genbank accession numbers have been added to the text.

Kind regards
Niels Daugbjerg.

Highlights.

- We established for the first time six clonal cultures of *Alexandrium tamarense* from west Greenland
- Based on partial LSU rRNA sequences, the Greenlandic cultures grouped within 'Group 1'
- Three cultures produced large amounts of gonyautoxins 1 and 4 (>60% total mol), the first record of saxitoxins in western Greenland
- We consider *A. tamarense* a likely agent for the PSP exceeding 800 µg toxin/kg shellfish (scallops)
- Toxin profiles were atypical for *A. tamarense* in their absence of N-sulfocarbanoyl C1/C2 or B1/B2

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

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Keywords: *Alexandrium tamarense*; Greenland; LC-FD; LC-MS/MS; LSU rDNA; PSP toxins; toxic dinoflagellates

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 *A. tamarense* morphotype, and bayesian and neighbour-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 towards 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).

Alexandrium 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 centred 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 (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 to 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.

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 Harbour (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 et al., 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 Centre 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. Polymerase chain reaction (PCR) amplification of partial LSU sequence (approximately 1500 bp) was performed in 39.2 µl PCR solution containing 4 µl of Taq buffer, 20 µl of 0.5 µM dNTP mix, 5 µl 10 µM of each primer, 5 µl 100 mM tetramethylammonium chloride, 0.1 µl 10 mg mL^{-1} of BSA (bovine serum albumin) and 0.1 µl of Taq-polymerase (Ampliqon, Herlev, Denmark). The amplification primers were D1R-F (Scholin et al., 1994) and 28-1438 (Daugbjerg et al., 2000). An initial denaturation step at 94 °C for 3 min, and 35 cycles, consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 52 °C and 3 min of elongation at 72 °C, was

142 followed by a final extension step at 72 °C for 10 min. Five µl of the PCR-produced LSU rDNA
143 fragments were loaded onto a 2% Nusieve ethidium bromide gel, run for 20 min at 150 mV and
144 examined under UV illumination to ensure that the amplifications were of the expected size. The
145 øX174 *Hae*III marker (ABgene, Rockford, IL, USA) was used for length comparison.
146
147 *2.3.2. DNA purification and sequencing*
148 DNA was purified by adding 50 µl of TE buffer to the PCR product and transferring the mix
149 to a well on a NucleoFast 96 PCR plate (MACHEREY-NAGEL, Düren, Germany). After applying
150 vacuum (ca. -0.5 bar) to the plate for 15 min, the DNA was recovered by adding 50 µl of double-
151 distilled H_2O to each well, mixing on a plate shaker for 10 min and pipetting of the dissolved DNA
152 into an Eppendorf tube. The concentration of dsDNA was measured using a BioPhotometer
153 (Eppendorf, Hamburg, Germany). The LSU rDNA sequences was determined in both directions
154 using the amplification primers and the primers D3A, D3B (Nunn et al., 1996) and D2C (Scholin et
155 al., 1994). Sequencing was performed at the facilities of Macrogen (Seoul, Korea). Genbank
156 accession numbers are provided as follows: K-0973 (JX155662), K-0974 (JX155664), K-0975
157 (JX155663), A1 (JX155665), D2 (JX155666), E1 (JX155667).
158
159 *2.3.3. Sequence alignment and phylogenetic analyses*
160 Phylogeny of the six novel partial LSU sequences was inferred after alignment with 81 other
161 partial LSU sequences from *Alexandrium* spp. retrieved from GenBank. Nineteen sequences were
162 from outside the *A. tamarense* species complex and served as outgroup. The alignment was done
163 with the ClustalW multiple alignment tool (Thompson et al., 1994) and further edited manually by
164 BioEdit v. 7.0.9.0 (Hall, 1999). As most of the retrieved sequences consisted only of the D1-D2
165 domains of the LSU, the alignment was trimmed at the 3' end, leaving a matrix of 647 base pairs

from which to infer a phylogeny. The matrix was analysed with Bio-Neighbor Joining (BioNJ) (Gascuel, 1997) using PAUP* v. 4.0b10 (Swofford, 2002) and Bayesian analysis (BA) with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). Modeltest v. 3.7 (Posada and Crandall, 1998) was used to reveal the best model for the LSU rDNA gene sequences by hierarchical likelihood ratio tests. The best model was TrN+I+G (Tamura and Nei, 1993) with among sites heterogeneity ($\alpha = 1.1791$), an estimated proportion of invariable sites ($I = 0.2146$) and two substitution-rate categories (A-G = 2.2611 and C-T = 4.5843). Base frequencies were set as follows A = 0.2686, C = 0.1521, G = 0.2530 and T = 0.3263. This model was applied to compute dissimilarity values, and the resulting distance matrix was used to build a tree with the BioNJ method. BioNJ bootstrapping invoked 1000 replications. Bayesian analysis was performed using a General Time Reversible (GTR) substitution matrix estimated from the data. A total of 2 million Markov Chain Monte Carlo (MCMC) generations with four parallel chains (one cold and three heated) was performed. By plotting the log likelihood values as a function of generations in a spreadsheet, the ln L values converged at -5,210 after 20,050 generations. This number of generations was used as the "burn in", resulting in 39,600 trees. They were imported into PAUP*, and a 50% majority rule consensus tree was constructed.

2.4. Toxin analysis

2.4.1 Liquid chromatography with fluorescence detection (LC-FD)

Between 3,000 and 100,000 cells were harvested in the late exponential phase by centrifugation (9,000 X g for 5 min), suspended in 1.0 ml of 0.03 M acetic acid, and transferred into a FastPrep tube containing 0.9 g of lysing matrix D (Thermo Savant, Illkirch, France). The samples were homogenized by reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France). After homogenization, samples were

centrifuged (Eppendorf 5415 R, Hamburg, Germany) at 16,100 X g at 4 °C for 15 min. The supernatant (400 µl) was transferred to a spin-filter (pore-size 0.45 mm, Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 800 X g. The filtrate was analyzed by reverse-phase ion-pair liquid chromatography with fluorescence detection (LC-FD) and post-column derivatisation following minor modifications of previously published methods (Diener et al., 2006; Krock et al., 2007). The LC-FD analysis was carried out on a LC1100 series liquid chromatography system consisting of a G1379A degasser, a G1311A quaternary pump, a G1229A autosampler, and a G1321A fluorescence detector (Agilent Technologies, Waldbronn, Germany), equipped with a Phenomenex Luna C18 reversed-phase column (250 mm X 4.6 mm id, 5 µm pore size) (Phenomenex, Aschaffenburg, Germany) with a Phenomenex SecuriGuard precolumn. The column was coupled to a PCX 2500 post-column derivatisation system (Pickering Laboratories, Mountain View, CA, USA). Eluent A contained 6 mM octanesulphonic acid, 6 mM heptanesulphonic acid, 40 mM ammonium phosphate, adjusted to pH 6.95 with dilute phosphoric acid, and 0.75 % tetrahydrofurane. Eluent B contained 13 mM octanesulphonic acid, 50 mM phosphoric acid, adjusted to pH 6.9 with ammonium hydroxide, 15 % acetonitrile and 1.5 % tetrahydrofurane. The flow rate was 1 ml min^{-1} with the following gradient: 0 – 15 min isocratic A, 15 – 16 min switch to B, 16 – 35 min isocratic B, 35 – 36 min switch to A, 36 – 45 min isocratic A. The injection volume was 20 µL and the autosampler was cooled to 4 °C. The eluate from the column was oxidized with 10 mM periodic acid in 555 mM ammonium before entering the 50 °C reaction coil, after which it was acidified with 0.75 M nitric acid. Both the oxidizing and acidifying reagents entered the system at a rate of 0.4 mL min^{1}. The toxins were detected by dual-monochromator fluorescence (λ_{ex} 333 nm; λ_{em} 395 nm). The data were processed with Agilent Chemstation software. Standard solutions of PSP toxins were purchased from the Certified Reference Material Programme of the Institute of Marine Biosciences, National Research Council, Halifax, NS, Canada.

214

215 *2.4.2. Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)*

216 Mass spectral experiments used an ABI-SCIEX-4000 Q Trap, triple quadrupole mass
217 spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. The LC
218 equipment included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A),
219 refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A).
220 Mass spectrometric analyses for PSP toxins were performed according to the hydrophilic interaction
221 liquid ion-chromatography (HILIC) method (Diener et al., 2007) with slight modifications. The
222 analytical column (150 × 4.6 mm) was packed with 5 μm ZIC-HILIC (SeQuant, Lund, Sweden) and
223 maintained at 35 °C. Flow rate was 0.7 mL min^{-1} and gradient elution was performed with two
224 eluants. Eluant A was 2 mM formic acid and 5mM ammonium formate in acetonitrile/water (80:20
225 v/v) and eluant B was 10 mM formic acid and 10 mM ammonium formate in water. The gradient
226 was as follows: 20 min column equilibration with 80% A, linear gradient until 5 min to 65% A,
227 then until 10 min to 60% A, then until 20 min 55% A, subsequent isocratic elution with 55% A until
228 24 min and finally return to initial 80% A until 25 min. Total run time was 45 min and the sample
229 volume injected was 5 μL. Selected reaction monitoring (SRM) experiments were carried out in
230 positive ion mode by selecting the following transitions (precursor ion > fragment ion), period 1 (B,
231 C- and gonyautoxins): *m/z* 412>332 and *m/z* 412>314 (for GTX1/GTX4 and C3/C4), *m/z* 396>316
232 and *m/z* 396>298 (for GTX2/GTX3, C1/C2 and B2), *m/z* 380>300 and *m/z* 380>282 (for B1), *m/z*
233 353>273 (for dcGTX2/dcGTX3), *m/z* 369>289 (for dcGTX1/dcGTX4); period 2 (STX, NEO and
234 their decarbamoyl derivatives): *m/z* 300>282 and *m/z* 300>204 (for STX), *m/z* 316>298 and *m/z*
235 316>196 (for NEO), *m/z* 257>196 and *m/z* 257>156 (for dcSTX) and *m/z* 273>255 (for dcNEO).
236 Dwell times of 100–200 ms were used for each transition. For these studies the following source

parameters were used: curtain gas: 30 psi, temperature: 650 °C, ion-spray voltage: 5000 V, gas 1 and 2: 70 psi, interface heater: on, collision gas: high, declustering potential: 66 V, entrance potential 10 V, collision energy: 30 V and collision cell exit potential: 12 V.

3. Results

3.1 Morphology

The three isolates examined under the light microscope all shared the morphological features of *Alexandrium tamarense* Lebour (Balech) *sensu* Balech (1995) (Fig. 2), i.e. the nearly spherical form slightly longer than wide with an average length/width ratio of 1.18 (n = 30). The length of the cells varied from 25 to 46 µm and the width from 20 to 40 µm (Table 2). The first apical plate (1') had a small ventral pore and the anterior sulcal (sa) plate had a shape typical of *A. tamarense*. A wide 6th precingular (6'') plate was also noted.

3.2 LSU rDNA analysis

In both the Bayesian and BioNJ analyses the six LSU rDNA sequences of *Alexandrium* from Greenland branched out in the recently defined Group I (Lilly et al., 2007) (formerly known as the North American clade, Scholin et al. 1994) of the *Alexandrium tamarense* species complex (Fig. 3). The tree presented is the 50% majority rule consensus tree from the Bayesian analysis. An identical tree topology was obtained in BioNJ. Posterior probabilities and bootstrap values ≥ 50 are plotted above and beneath the branches, respectively. The novel sequences branched out together, and the calculated distance matrix (Table 3) revealed that K-0975 differed from the other five Greenland isolates by substitution of a single base pair (pos. 224 of the submitted sequence). These five were

261 identical in LSU rDNA sequences to nine other strains belonging to Group I and distributed from
262 South Korea to South Africa (Lilly et al. 2007).
263
264 *3.3 Toxin composition*
265 All *A. tamarense* isolates analyzed from Greenland contained saxitoxin or derivatives
266 thereof (Fig. 4, Table 4) and were characterized by high percentages of the gonyautoxins
267 GTX1/GTX4. Although the epimers GTX1 and GTX4, and GTX2 and GTX3, were analytically
268 separated, they are presented (Fig. 4) as epimeric pairs due to facile interconversion resulting from
269 thermodynamic equilibrium. The isolates K-0973, K-0974, and K-0975 exhibited a similar toxin
270 profile (but not virtually identical molar composition) composed of high GTX1/GTX4 (>60 mol%),
271 with lesser proportions of GTX2/GTX3, neosaxitoxin (NEO) and STX (Fig. 4). No trace of either
272 *N*-sulfocarbamoyl (B1/2, C1 - C4) or decarbamoyl (dcSTX, dcNEO, dcGTX 1 - dcGTX4) toxins
273 were detected in these isolates. All isolates were analysed separately at least twice from
274 exponentially growing cultures because in the first round the number of cells extracted was not
275 reliably counted and thus only the molar percentage of the toxins was obtained. Isolate K-0974 was
276 analyzed more thoroughly than the others as STX and NEO were close to the detection limit. After
277 increasing the number of extracted cells for this isolate, NEO was detected again, whereas STX was
278 not. The cell toxicity of the three isolates, calculated as STXeq $cell^{-1}$ according to toxicity factors
279 given in Oshima (1995), ranged from 10.3 to 16.8 pg STXeq $cell^{-1}$.
280 The identification of PSP toxins in our isolates of *A. tamarense* from Greenland based on
281 LC-FD (i.e. Fig. 4 and Table 4) was confirmed unambiguously by liquid chromatography with
282 tandem mass spectrometry (Krock et al. 2007). The two methods revealed quantitative differences
283 in PSP toxin content per cell among the isolates (Table 5) and LC-FD and LC-MS/MS
284 independently verified the presence of the principal toxins GTX4, GTX1, GTX3 and NEO.

4. Discussion

Based on the overall morphological characteristics and plate tabulations of the three isolates from Greenland they clearly belong to the *Alexandrium tamarense* morphotype. The ventral pore on the first apical (1') plate is also present in *A. minutum* but the shape of the sixth precingular (6'') as well as the sa plate are tamarensoid. Although the general size and shape of the cells are more similar to *A. tamarense* than *A. minutum* these characters are variable (Balech, 1995) and thus can only be used as a first guide. Length (l) and width (w) as well as the l/w ratio were quite stable within the isolates but one isolate (K-0973) produced somewhat larger cells (Table 2). The species *A. ostenfeldii* often found in North Atlantic, North Sea and Scandinavian coastal waters is ruled out as an affiliation for any of the Greenland isolates by the absence of the characteristic large globose cell shape and the lack of a large kidney-shaped ventral pore at the margin of the 1' plate.

Large differences in size may be attributed to different stages in the life cycle – vegetative cells, gametes, planozygotes (Balech, 1995). However, the size variation within the strains is quite low, indicating that the cultures likely consist almost exclusively of vegetative cells.

The *Alexandrium* isolates from Greenland examined by molecular phylogenetic characteristics in this investigation all emerged in a clade previously known as the North American clade (Scholin et al. 1994), as part of the *A. tamarense* species complex within the newly defined Group 1 (Lilly et al., 2007), for lack of a better term. The known distribution of the strains in this group extends from the northeast Atlantic westward around the Americas to the northwest Pacific. Not surprisingly the strains from Greenland belong to this group, and we now confirm that toxigenic members of this clade occur in arctic waters. Whether the species is a new arrival in the Arctic, either due to natural or human mediated dispersal, or if the late discovery reflects a paucity

of observations is unknown, but we are not aware of *A. tamarense* having been observed previously in Greenland waters.

Numerous previous investigations of PSP toxin variation among *Alexandrium* species and populations (reviewed by Anderson et al. 1994; Cembella 1998; Alpermann et al. 2010) have indicated that toxin profiles are genetically determined and stable enough (within limits of physiological variation under defined conditions) to serve as a phenotypic marker. The fact that Greenland isolate K-0974 produces more than 98 mol% of 1-N-hydroxy (R1 = -OH) toxins may also be helpful for elucidation of the biosynthetic pathway of these toxins. However, the toxin profiles of the Greenland isolates are rather unusual and atypical for *A. tamarense*. One unusual feature is the complete absence of N-sulfocarbamoyl C1/C2 or B1/B2 toxins, which are usually present in most strains of the *A. tamarense* species complex, often in a high molar percentage (Cembella et al., 1987; Anderson et al., 1994; Persich et al., 2006; Krock et al., 2007; Orlova et al., 2007). The high molar percentage of GTX1/GTX4 toxins (> 60 mol%) and lesser amounts of only carbamoyl toxins, including GTX2/3, NEO or STX, plus the absence of decarbamoyl derivatives are more typical of strains of *A. minutum* (Franco et al., 1994; Hwang and Lu, 2000; Carreto et al., 2001; Hansen et al., 2003; Chou et al., 2004; Pitcher et al., 2007). The toxin profile of K-0974 with the almost exclusive production of GTX1/GTX4 (> 95 mol%) is similar to that reported from strains NEPCC 253 from Laguna Obidos, Portugal and NEPCC 508 from Whangarei, North Island, New Zealand and originally assigned to the NEP Culture Collection as members of the *A. tamarense* species complex (see Table 4) (Cembella et al., 1987). One small difference is the detection of NEO in K-0974, whereas this component is absent from the Portuguese and New Zealand isolates.

No LSU rDNA sequences or other molecular markers are available for these latter strains, but it is unlikely that they are closely related to K-0974. In any case, subsequent careful

morphological analysis of thecal plates of NEPCC 253 and NEPCC 508 (A. Cembella, unpublished observations) indicate that both strains belong to the *A. minutum* sub-group. NEPCC 508 accords best with the description of *A. angustitabulatum* (unusually narrow 6" plate).

To our knowledge, previous molecular data on *Alexandrium* phylogenetic affiliations from high latitude oceans are limited to a single strain of *A. tamarense* (Group 1) of unknown toxicity from the Faroe Islands (Lilly et al. 2007). We show here that *Alexandrium* from Greenland are toxic and provisionally conclude that *A. tamarense* is likely the primary contributor to PSP toxicity in scallops in the Attu area. *Alexandrium ostenfeldii*, another potential PSP toxin producer, was also found in the area (Ø. Moestrup, personal observation), but, with the exception of the Baltic Sea, in northern Europe this species has never been known to produce dense blooms. Furthermore, isolated strains from the North Sea and North Atlantic tend to produce the macrocyclic imine toxins spirolides and only little (if any) PSP toxins (MacKinnon et al. 2006). *Alexandrium minutum* of unknown toxicity has been found in the Disko Bay area further north (Jensen and Veland, 2006) and although not seen in the Attu area it could be present cryptically and contribute to PSP toxicity in scallops.

Concerns have been expressed that rising global temperatures could lead to a northward range extension and/or increase in endemic HABs in arctic areas. This could include blooms of *Alexandrium* spp. along the Greenland coast. We noted that the Greenland isolates grew very slowly in culture when incubated at 4 °C, approximately the ambient sea temperature of their natural habitat, but shifted up growth rates dramatically at higher temperatures (i.e. 10 °C). Since PSP toxin cell quota is generally positively correlated with growth rate in *Alexandrium* spp. (reviewed in Cembella 1998), any major rise in sea temperature offers the possibility of both higher magnitude toxic blooms and increased cell potency. Under present circumstances in Greenland, this also provokes the question of how the current *Alexandrium* populations generate enough toxins to cause

toxicity in the scallops, even in some cases beyond the regulatory limit. We are not certain that under ambient nutrient and light regimes (e.g., long day length in summer) in nature, that the low growth rates we achieved in culture at low temperatures are representative. Furthermore, under low temperatures the reduced metabolic rates in bivalve molluscs would be expected to cause scallops to retain the toxins for longer periods (Bricelj and Shumway, 1998).

5. Conclusions

The LSU sequences clearly place the isolates from Greenland of *Alexandrium tamarense* within Group 1 of the *A. tamarense* species complex as defined by Lilly et al. (2007). One of the six sequences differed from the others by a single substitution, indicating one large homogeneous population of *A. tamarense* along the west coast of Greenland. Further genetic assays, microsatellite or amplified fragment length polymorphisms (AFLP), will be helpful in elucidating further the population structure of the *A. tamarense* species complex from Greenland. The toxin profiles of the three cultured strains, with large molar percentages of GTX1/GTX4, are closer to the toxin profile of *A. minutum* than to that of members of the *A. tamarense* species complex. The latter group is usually characterised by a high percentage of N-sulfocarbamoyl (C1/C2) toxins. Additional strains of the *A. tamarense* species complex from Greenland as well as other areas in the Arctic should be established to determine whether the unusual toxin profiles are a common feature of arctic strains or if they represent a local or regional anomaly. Natural blooms of members of the Group I clade of *A. tamarense*, represented by the three strains established here, must be considered as the most likely agents for PSP toxin accumulation in the scallops from western Greenland, but no toxin profiles are available from the contaminated bivalves or other putatively toxic *Alexandrium* species from this region. Therefore, an effort should be made to obtain *A. ostenfeldii* and *A. minutum* in culture, as these species have also been observed along the west coast of Greenland.

Acknowledgements

We thank the ATI School in Maniitsoq for arranging the visit of ØM to Greenland and the sampling cruise. Helle Siegstad, (Greenland Institute of Natural Resources, Nuuk) and Bjarne Ring Thorbjørnsen (Danish Food and Veterinary Administration, Viborg) generously provided access to data. Lene Christiansen (Department of Biology) provided laboratory assistance and Annegret Müller (AWI, Bremerhaven) performed toxin chromatographic analysis.

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Figure legends

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.

Fig. 2. Micrographs of *Alexandrium tamarense* from the west coast of Greenland. Scale bars = 10 µm. A-C: K-0975. (A) Ventral view of cell showing cingulum (c) and sulcus (s); (B) Epicone showing 4 (1' -4') apical plates with a ventral pore (vp) on 1' and 6 (1'' – 6'') precingular plates; (C) Anterior sulcal plate (sa). D-F: K-0973. (D) General shape of the cell, slightly longer than wide; (E) Epicone with vp clearly visible on 1', the apical pore (po) complex is seen in the centre; (F) Sa plate and po in the centre. G-I: K-0974. (G) Dorsal view with cingulum (c) visible on both sides; (H) Epicone with vp on 1' and po in the centre; (I) Epicone plates with sa.

Fig. 3. Phylogeny the *Alexandrium tamarense* species complex based on partial nuclear-encoded LSU rDNA sequence and inferred from Bayesian analysis. The alignment included 647 nucleotides. Branch lengths are proportional to the number of substitutions per site. At internal nodes posterior probabilities (≥ 0.5) are listed first followed by bootstrap values (≥50%) from BioNeighbor-joining analyses. Isolates from Greenland are in bold face.

Fig. 4. Toxin composition of *Alexandrium tamarense* from the west coast of Greenland expressed as mol% of total toxins. Error bars represent standard deviations.

523 **Tables**

524 Table 1

525 Location, coordinates and dates of collection. The strains/isolates K-0973, K-0974 and K-0975 are
526 available from Scandinavian Culture Centre 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

527

528 Table 2

529 Length (µm), width (µm) and L/W ratio of the three strains of *A. tamarense* from Greenland. n =
530 10. Numbers in brackets are standard deviations.

Strain	Length	Width	L/W ratio
K-0973	33.10 (6.53)	28.01 (5.95)	1.19 (0.09)
K-0974	33.69 (2.04)	29.75 (2.35)	1.13 (0.06)
K-0975	42.16 (3.24)	35.20 (3.15)	1.20 (0.10)

531

532 Table 3

533 Absolute distance matrix of 647 base pairs from the domain D1 to D2 of the LSU rDNA gene.
534 Numbers indicate that K-0975 has 1 base pair substitution compared to the other five isolates

	K-0973	K-0975	K-0974	A1	D2	E1
K-0973	–					
K-0975	1	–				
K-0974	0	1	–			
A1	0	1	0	–		
D2	0	1	0	0	–	
E1	0	1	0	0	0	–

535

Table 4

PSP toxin concentration and composition of *Alexandrium tamarense* cultures from Greenland determined by LC-FD. Numbers in brackets are standard deviations

Strain		Toxin						
		STX	NEO	GTX 1	GTX 2	GTX 3	GTX 4	Total
K-0973 (Attu)	fmol cell^{-1} [c]	2.35 (1.21)	7.63 (4.05)	1.60 (3.20)	0.40 (0.18)	4.75 (1.12)	39.79 (14.89)	
	fg cell^{-1} STXeq [c]	707.20 (364.06)	2226.93 (1183.01)	652.40 (1304.79)	57.34 (25.62)	1204.08 (283.55)	11978.81 (4481.10)	16827
	mol% comb. epimers [a d]	6.10 (3.61)	14.73 (3.86)	65.27 (12.92)	13.90 (7.23)			
K-0974 (Attu)	fmol cell^{-1} [e]	0.00 (0.00)	0.28 (0.48)	0.97 (1.67)	0.00 (0.00)	0.41 (0.31)	32.21 (4.98)	
	fg cell^{-1} STXeq [e]	0.00 (0.00)	81.76 (139.63)	397.04 (681.28)	0,00 (0.00)	103.62 (79.10)	9695.34 (1497.67)	10278
	mol% comb. epimers [a f]	0.08 (0.15)	1.87 (2.52)	96.40 (3.53)	1.65 (1.22)			
K-0975 (Maniitsoq)	fmol cell^{-1} [c]	1.07 (0.18)	3.74 (0.20)	0.00 (0.00)	0.37 (0.12)	10.51 (1.69)	34.75 (9.50)	
	fg cell^{-1} STXeq [c]	323.63 (53.82)	1091.71 (57.98)	0.00 (0.00)	53.03 (17.60)	2665.63 (427.53)	10461.48 (2858.35)	14595
	mol% comb. epimers [a d]	2.16 (0.47)	7.18 (1.77)	62.10 (10.14)	28.56 (10.62)			
NEPCC 253 [b] (Portugal)	fmol cell^{-1} comb. epimers [a]			1.13 (0.13)	0.03 (0.01)			
	mol% comb. epimers [a]			97.41 (2.24)	2.59 (2.24)			
NEPCC	fmol cell^{-1}	0.02		2.66 (1.17)	0.08 (0.04)			

508[b] (New Zealand)	comb. epimers[a]	(0.02)			
	mol% comb. epimers[a]	0.72 (0.71)		96.38 (2.79)	2.90 (1.45)

[a]Combined epimer pairs are: GTX 1 + GTX 4, GTX 2 + GTX 3. [b]Data from Cembella et al. 1987. [c]n=4. [d]n=6. [e]n=7 [f]n=9.

Figure 1

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Figure 2

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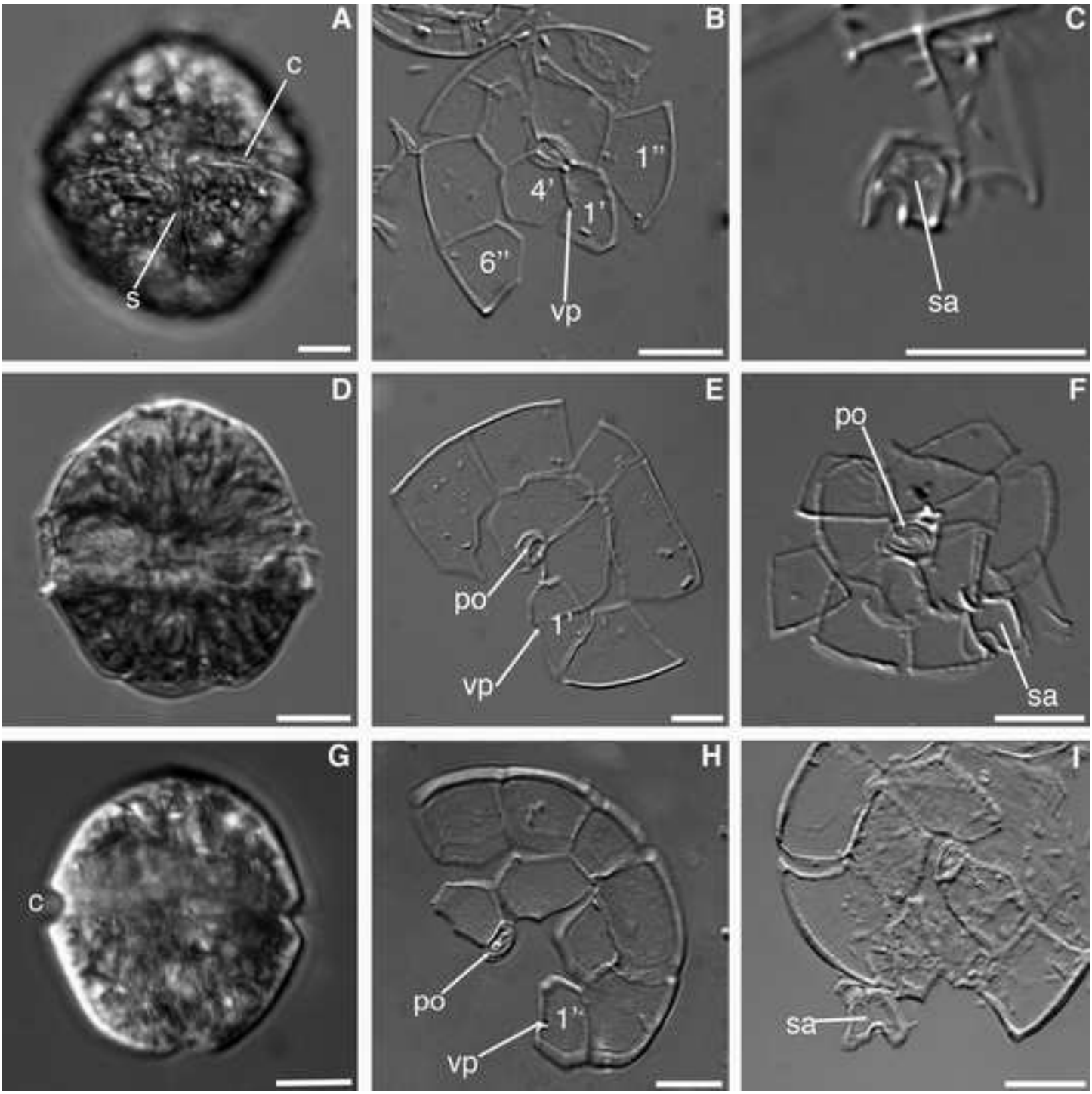

Figure 3

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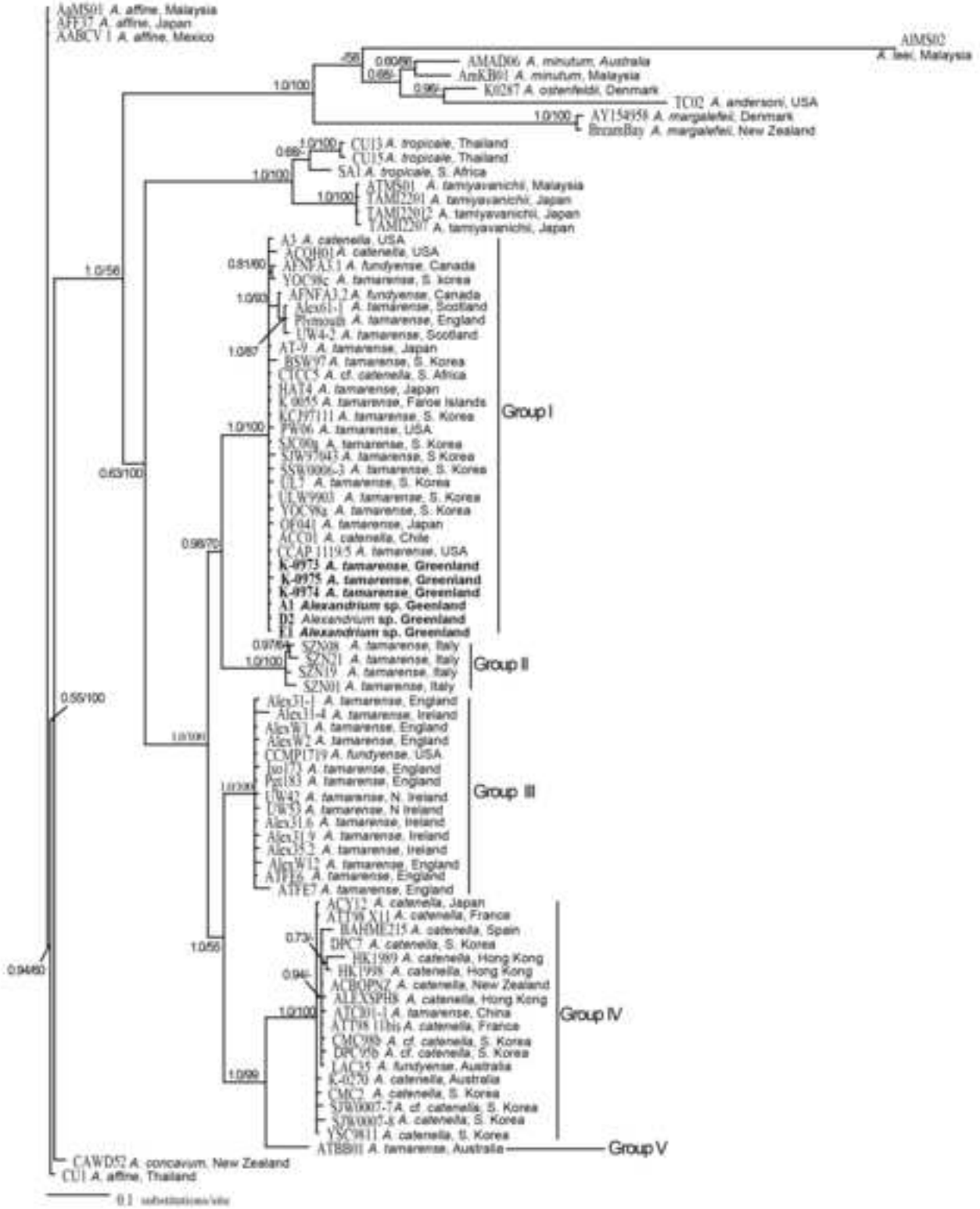

Figure 4

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