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First report of goniodomin A production by the dinoflagellate *Alexandrium pseudogonyaulax* developing in southern Mediterranean (Bizerte Lagoon, Tunisia)

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Abstract :

The dinoflagellate *Alexandrium pseudogonyaulax* is widely distributed around the world including the Mediterranean waters. The objectives of this study were to determine the morphology and phylogenic affiliation of *A. pseudogonyaulax* strain isolated from Bizerte Lagoon (Mediterranean waters, Tunisia) and investigate its toxicity. Molecular analyses confirmed the morphological identification of the isolated strain (APBZ12) as *A. pseudogonyaulax.* Moreover, it showed that it is 100% identical with strains of this species found in New Zealand, Japan, China and North Sea (Norway and Denmark) suggesting that this species is cosmopolitan. Until now, no toxin studies have been conducted on fully characterized (morphologically and molecularly) *A. pseudogonyaulax.* Cellular toxin production was determined using high pressure liquid chromatography coupled to mass spectrometry (HPLC/MS). Results showed for the first time that *A. pseudogonyaulax* contains goniodomin A (GDA), a highly toxic macrolide polyether previously shown to be produced by two other dinoflagellate species *Alexandrium monilatum* (Hsia et al., 2006) and *Alexandrium hiranoi* (erroneously identified as *A. pseudogonyaulax* in Murakami et al., 1988) in American and Japanese waters, respectively. This biologically active toxin has been associated over decades with fish mortality. Our study showed that the cell extracts of APBZ12 showed an important bioactivity using GH4C1 rat pituitary cytotoxicity bioassay.

Highlights

1 *A. pseudogonyaulax* cysts have different morphotypes
2 Phylogenetic tree showed that this species is cosmopolitan.
2 GDA was exracted from *A. pseudogonyaulax* vegetative cells for the first time
3 *A. pseudogonyaulax* cell extract showed an important cytotoxicity

Keywords : *Alexandrium pseudogonyaulax*, goniodomin A, Mediterranean sea, Morphology, Phylogeny

1. INTRODUCTION

Human health and the shellfish industries are increasingly and seriously threatened by toxins produced by harmful algal bloom (HAB) species. *Alexandrium* (Dinophyceae) is one of the major HAB genera considering its diversity, wide distribution and impact on the ecosystem and aquaculture activity. It presents an important toxigenic diversity by producing three different toxin families: Saxitoxins, Spirolides and Goniodomins (Anderson et al., 2012). Goniodomin A (GDA) is a macrolide polyether, with important biological properties and is responsible for human illness and death of aquatic fauna. It has been shown that this toxin affects human cytoskeleton reorganization (Takeda et al., 2008), human brain cells, cardiac functioning (Mizuno et al., 1998, Matsunaga et al., 1999) and it also inhibits regeneration of blood vessels (angiogenesis) (Abe et al., 2002). Exposure to GDA resulted in paralysis and death of fish and mollusks (Sievers, 1969, Harding et al., 2009) after 24–48 h of exposure and was preceded by external signs of stress including reduced ventilation, inability to attach to hard substrates, periodic pumping of the opercular plate, and increased mucus production**.** It has been shown that the chain-forming dinoflagellate *Alexandrium monilatum* was probably associated to widespread discolored water and increased fish mortality in the Mississippi Sound and off the eastern and western coasts of Florida over 60–70 years (Howell, 1953). Hsia et al. (2006) by utilizing combined NMR spectroscopy and mass spectrometry structurally characterized GDA in *A. monilatum* cells. This toxin was shown to be released when cells were stressed or ruptured in response to mechanical stimulation as fresh water precipitation or a bloom termination (Connell and Cross 1950, Gates and Wilson 1960). Sharma et al. (1968) reported the isolation of GDA, an antifungal compound, from the dinoflagellate *Goniodoma* sp. which bloomed in a bay near la Paraguera, Puerto Rico. Murakami et al. (1988) isolated, in 1986, the active substance GDA from a rock pool at Jogashima (Japan) from a dinoflagellate identified by Kita et al. (1985) as *Alexandrium*

pseudogonyaulax (syn. *Goniodoma pseudogonyaulax*). However, Kita and Fukuyo (1988) considered that this GDA-producing dinoflagellate in Jogashim was misidentified as *Alexandrium pseudogonyaulax* and described it as *Alexandrium hiranoi* Kita and Fukuyo.

Alexandrium pseudongonyaulax is a brackish-neritic dinoflagellate that belongs to the subgenus *Gessnerium* (Balech, 1995). This species was originally described as *Goniodoma pseudogonyaulax* Biecheler from the Thau lagoon (Mediterranean, France) (Biecheler, 1952). This dinoflagellate is distributed worldwide including the Mediterranean Sea, Japanese coastal waters and the Gulf of Georgia in British Columbia (Biecheler, 1952, Montresor, 1995, Bravo et al., 2006, Daly Yahia-Kéfi et al., 2001, Turki, 2004, Klein et al., 2010) and has also been reported as a bloom-forming in the Norwegian Sea (Throndsen et al., 2007) and in Alfacs Bay (Bravo et al., 2006). Klein et al. (2010) mentioned that *A. pseudogonyaulax* could be responsible of harmful effects to high marine trophic levels but the toxigenic potential of this species remains unknown since no toxicological analyses nor toxin extraction and identification have been performed on fully characterized *A. pseudogonyaulax* strains. To our knowledge, no formal studies were undertaken on determination of *A. pseudogonyaulax* toxicity showing the presence of GDA. Zmerli Triki et al. (2014) reported that the average *A. pseudogonyaulax* resting cyst density across the whole Bizerte lagoon (Southern Mediterranean, Tunisia) was rather high, reaching 639 cysts g^{-1} of dry sediment (DS) suggesting that this species developed regularly in this lagoon. Cyst germination could initiate important toxic blooms when environmental conditions become favorable which in turn could affect marine ecosystem components and cultivated mollusks in this area. The present study aimed to 1) fully characterize *A. pseudogonyaulax* originating from Bizerte lagoon using morphological examination and ribotyping, 2) analyze the toxin content of this dinoflagellate by HPLC/MS and 3) investigate the bioactivity of *A. pseudogonyaulax* cell extract.

2. MATERIAL & METHODS

2.1. Sediment sampling and natural resting cyst extraction

Sediments were sampled by scuba divers using core samplers in July-August 2012 in Bizerte lagoon (37º 8'- 37º14' N, 9º46'-9º56' E) North-East Tunisia (Fig. 1). The surface layer of sediment cores (3 cm) was sliced and stored at 4 °C in the dark for several days before processing. For cyst extraction, one gram of wet sediment was suspended in 50 mL of Filtered Sea Water (FSW) and sonicated for 3 min. The mixture was sieved under 100 µm and 20 µm mesh because *A. pseudogonyaulax* cyst diameter was above 20 µm. The slurry remaining on the 20 µm mesh was recovered with FSW, then centrifuged (MIKRO 22R HETTICH centrifuges) for 10 min at 4 °C and 3000 $t.min^{-1}$. Cysts were extracted from the resulting pellet using density gradient method with Polytungstate Solution (PST) (Bolch, 1997). Cysts recovered in the supernatant phase after PST centrifugation were flushed several times with FSW to remove PST then stored in dark at 4 °C.

2.2. Morphological identification of A. pseudogonyaulax resting cysts

Resting cysts (RCs) identification was based on microscopic observation of the morphology of the cysts and resulting vegetative cells obtained from excystment experiments. *Alexandrium pseudogonyaulax* RCs were isolated into 96-culture plates (Nunc™ Delta surface) filled with 200 µl of Enriched Natural Sea Water (ENSW) culture medium (Harrison et al., 1980) and incubated at 20 °C, salinity of 34, 100 µmol photons m^{-2} s^{-1} and 12 h: 12 h light:dark ratio. When germination occurred, the morphology of the emerging vegetative cells and empty cysts were examined using a light microscope and 100X magnification (Esselte Leitz GmbH, Germany). Within the culture, the obtained gametes and the planozyogtes were

also photographed. Data on the life cycle of this dinoflagellate and detailed biometry of different stages was published in Zmerli Triki et al. (2015).

2.3. Algal cultures

One monoclonal culture of *Alexandrium pseudogonyaulax* was obtained from the germination of a wild resting cyst (strain APBZ12) and used for molecular identification and toxin characterization, this culture provided relatively high cell densities required for the subsequent analyses. The stock culture was maintained in sterile flasks filled with 200 mL of ENSW medium at stable conditions of salinity 34, temperature 20 °C, irradiance of 100 µmol photons m^{-2} s^{-1} using cool white fluorescent light on a 12:12 light:dark cycle.

2.4. Molecular identification and phylogeny

2.4.1. DNA extraction and PCR

Total genomic DNA was extracted from the strain APBZ12 (centrifuged pellets of 100 ml cultures; 60 cell.ml^{-1}) using the classical phenol-chloroform method (Sambrook et al., 1989). Cellular material was released by enzymatic lysis using proteinase K digestion. The DNA was separated from protein by phenol:chloroforme:isoamyl alcohol (25:24:1) extraction then extracted using chloroform:isoamyl alcohol (24:1). The separation of the aqueous and organic phases was performed by centrifugation. The aqueous phase contains the DNA which was ultimately recovered in solid form as a result of precipitation in ethyl alcohol. DNA was then resuspended on ultra pure water. For PCR, the oligonucletide primers and methods used were those described in Nézan et al. (2014).

145 ***2.4.2. Phylogeny***

146 For the phylogenetic analysis, the sequence of the Bizerte strain (APBZ12) was aligned
147 together with 41 LSU sequences of other *Alexandrium* species and one sequence of
148 *Goniodoma polyedricum* (as outgroup) retrieved from GenBank using the multiple sequence
149 alignment program MUSCLE version 3.7 (Edgar, 2004). The alignment was refined by eye.
150 The data matrix obtained (42 LSU rDNA sequences, 657 characters) was analyzed by two
151 methods of phylogenetic reconstruction: maximum likelihood (ML), using PhyML v.3.0
152 software (Guindon and Gascuel, 2003) and Bayesian inference (BI) using MrBayes v.3.1.2
153 (Ronquist and Huelsenbeck, 2003). The software jModeltest v 0.1.1 (Posada, 2008) was first
154 used to select the most suitable model of substitutions. The General-Time Reversible model
155 (GTR + I + G) was chosen as indicated by the Hierarchical Likelihood Ratio Tests (hLRTs),
156 Akaike Information Criterion 1 (AIC1), Akaike Information Criterion 2 (AIC2) and Bayesian
157 Information Criterion (BIC) tests implemented in jModeltest. Bootstrap values (support for
158 branches) of trees were obtained after 1000 iterations in ML. For Bayesian inference, four
159 Markov chains were run simultaneously for 2×10^6 generations with sampling every 100
160 generations. On the 2×10^4 trees obtained, the first 2000 were discarded (burn-in) and a
161 consensus tree was constructed from the remaining trees. The posterior probabilities
162 corresponding to the frequency with which a node is present in preserved trees, were
163 calculated using a coupled Monte Carlo Metropolis approach – Markov Chain (MCMC).

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165 ***2.5. Goniodomin A extraction, bioactivity and identification***

166 ***2.5.1. Extract preparation***

167 *Alexandrium pseudogonyaulax* culture was harvested in the exponential phase of growth,
168 approximately 12 days after the start of batch culture. The cells were centrifuged (5000 g, 4
169 min) and the pellets frozen at -20 °C before shipping to the NOAA Hollings Marine

Laboratory Emerging Toxins Program (Charleston, SC). For toxin extraction/identification and bioactivity testing, the pellets containing *A. pseudogonyaulax* cells were sharp frozen and lyophilized. The residual cell powder was extracted using an elutropic series of solvents of increasing polarity: 1) Acetone, 2) Methanol and 3) Water. All were taken to dryness prior to HPLC and or TLC purification/ analysis and then re-suspended in ethyl acetate (EtOAC) for loading on the TLC

2.5.2. Preparative thin layer chromatography (TLC)

The crude concentrated ethyl acetate extract of *A. pseudogonyaulax* was loaded on Whatman Silica Gel 60A preparative TLC glass-backed plate and then developed in a mixture of 7:3 ethyl acetate:benzene. All colored and non-colored bands were scraped from the plate, eluted with ethyl acetate, concentrated and tested for cytotoxicity. These bioassays used methanol as a carrier against the GH4C1 rat pituitary cells and an MTT dye as determining the endpoint (Mosmann, 1983). This procedure both established the Rf value of the toxic compound and effectively separated the toxin from contaminating pigments. A modified molybdic acid stain (Stahl, 1965; H_2SO_4) was used in addition to cytotoxicity testing in order to associate activity to colored and/or non-colored bands

2.5.3. GH4C1 rat pituitary cytotoxicity bioassay

Bioactivity was determined only from the acetone extract using the GH4C1 bioassay (Mosmann 1983) described in Hsia et al. (2006). Determination of cytotoxicity was carried out following the procedure in Hsia et al. (2006) using rat pituitary cells GH4C1 (ATCC CCL-82.2) in a MTT colorimetric assay (Manger et al., 1993; Mosmann, 1983). GH4C1 rat pituitary cells were plated onto a 96- well plate and allowed to incubate for 18h to allow for adherence to the plate. Dried fractions to be tested were solubilized in 100 µL methanol and

sonicated to ensure maximum solubility prior to carrying out the bioassay. The assay was carried out by adding 4 µL of the methanol-solubilized fractions to individual wells in the 96-well plate; 4 µL or less of pure methanol has been previously tested to be safe to these cells (i.e., a negative control). The plate was then allowed to incubate for a minimum of 18 h; 15 µL of 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye was added after incubation and allowed to incubate for another 4 h before adding 100 µL of SDS to each well. The plate was allowed to stand for another 4 h prior to reading the assay.

2.5.4. Goniodomin A identification

The bioactive fraction(s) were syringe filtered and then subjected to HPLC/MS purification and analyses to separate, detect and identify toxic extracts from the natural pigmented extracts produced from *A. pseudogonyaulax*. HPLC/MS purification and analysis was carried out using a WATERS HPLC system (WATERS 2767 Sample Manager), 1525 Binary Pump, 510 pump, WATERS 2996 PDA and a WATERS ZQ Single Quadrapole Mass Detector outfitted with an active flow splitter, switching valve and using MASS LYNX software (Waters Corporation, Milford, MA). The HPLC chromatography was performed using a Luna C18 column (2.5 µ particle size, 2.0 x 100 mm) (Phenomenex, Torrance, CA) using a reverse-phase water/ACN gradient with 0.1% TFA in each solvent. The HPLC/MS data (retention time and mass m/z (M+H: 769) was identical to that of goniodomin A as reported in Hsia et al. (2006). Confirmation was carried out by treating the toxic extract with NaCl and re-assessing the MS data. As predicted the sodium salt adduct of GDA (M+NA) was generated almost quantitatively. Accurate mass detection of the *A. pseudogonyaulax* toxic extract was performed using direct infusion of the sample into a Waters Micromass Quadrupole Q-TOF mass spectrometer operating with electrospray ionization in positive ion mode. KBr and NH4

were added in order to test for adduct formation. Chemical formulae were calculated utilizing the elemental composition tools within the Waters Masslynx software.

3. RESULTS

3.1. Morphology of Alexandrium pseudogonyaulax

Observed under the light microscope, the resting cysts (RCs) of *A. pseudogonyaulax* from Bizerte lagoon sediment exhibit different sizes and wall thickness. This variability can result in underestimation when quantifying these cysts. More than 200 RCs of *A. pseudogonyaulax* with different morphologies were incubated to ensure the reliability of our determination of *A. pseudogonyaulax* cysts (Fig. 2). RCs were slightly spherical with one flattened side (Fig. 2A, B). Cyst content was mostly dark and vesicular (Fig. 2C). We also observed clear cysts with brownish-yellow vesicular content (Fig. 2D) with the characteristic red body of *Alexandrium* species. The cyst wall can be paratabulated or smooth, thin or large (Fig. 2E, F, G). Cysts may produce a diploid planomeiocyst, or two or four haploid vegetative cells (Fig. 2H, I, J, K). Cysts diameter ranged from 40 to 72.5 µm (n = 200) with an average of 62.5 µm (SE ± 3.65). Laboratory produced cysts had vesicular dark content (Fig. 2P).

Excysted vegetative cells of *A. pseudogonyaulax* are thecate, medium to large in size, irregularly pentagonal-shaped with moderate dorso-ventral flattening. Cells are wider than long; the epitheca is slightly shorter than the hypotheca (Fig. 2M). Mean width ranged between 40.9 µm and 46.9 µm (Zmerli Triki et al., 2014). In the culture, smaller cells were identified as gametes while larger ones corresponded to planozygotes with two longitudinal flagella (Fig. 2N, O). The examination of the plate formula of the strain APBZ12 attested of *A. pseudogonyaulax* (Biecheler) Horiguchi ex Yuki et Fukuyo. The first apical plate (1') is characteristically displaced with a large ventral pore on the anterior margin. Thecal plates are smooth and thin with scattered minute pores, and their shape and pattern are designed in Fig.

3 and are in agreement with the description of *A. pseudogonyaulax* according to Balech (1995).

3.2. Ribotyping and phylogeny

A sequence of 703 base pairs has been obtained from the APBZ12 strain, comprising the partial LSU rDNA (D1-D2) and deposited in Genbank with the accession number KT229568. This sequence was similar with a batch of sequences from New Zealand, Japan, China, Norway and Denmark available in Genbank and all identified as *A. pseudogonyaulax*. The phylogeny inferred from LSU rDNA showed that all these sequences clustered in a highly supported clade which indicated that the LSU of the strain from Bizerte lagoon is identical to the strains also found in New Zealand, Japan, China and in North Sea (Norway and Denmark) (Fig. 4).

3.3. Bioactivity and toxin identification

Purification using preparative thin layer chromatography separated the toxic component from pigments also produced by *A. pseudogonyaulax*. Pigments such as peridinin and xanthophylls are cytotoxic in high concentrations. This extract was tested positive against the GH4C1 rat pituitary cell line cytotoxicity bioassay. The active compound(s) were eluted from the preparative TLC plate and taken for further purification and characterization on MS and LC–MS. The molecular ion of GDA (m/z M+H 769) (Fig. 5A) is clearly demonstrated in the active fraction. The HPLC retention time (not shown), and mass (m/z M+H 769) were identical to that of GDA as reported in Hsia et al. (2006). Active fraction shown in Fig. 5B was treated with excess NaCl generating the (m/z M+Na) sodium adduct of GDA 791.4 amu, as predicted and reported in Hsia et al. (2006). All mass spectra were obtained in positive ion mode. The chemical formula of GDA is shown in Fig. 6.

4. DISCUSSION

Morphological and molecular analyses performed on APBZ12 strain confirmed the identification of *Alexandrium pseudogonyaulax*. Moreover, it showed that this strain originating from Bizerte (Mediterranean) is 100% identical with strains of this species isolated from New Zealand, Japan, China and North Sea (Norway and Denmark). *Alexandrium pseudogonyaulax* therefore occurs worldwide including a semi-enclosed South-Western Mediterranean lagoon (Bizerte). This wide distribution could be explained by natural or human assisted dispersion throughout resting cysts transport by water ballast and/or shellfish (Hallegraeff, 1993, Laabir et Gentien, 1999) or/and the large geographical occurrence of an endogenous species. The life cycle of *A. pseudogonyaulax* showed a succession of different stages with distinctive morphology, physiology and function. Asexual reproduction was characterized by eleutheroschisis where a vegetative cell sheds its theca when settling on surface sediment, thereafter the resulting temporary cyst divides. Two types of conjugation of the gametes were observed in *A. pseudogonyaulax* including engulfment and fusion either with anisogamy or isogamy leading to the planozygote (Zmerli Triki et al., 2015). The morphology of vegetative cells was consistent with the general features of the species and they are described with more details in Zmerli-Tiki et al. (2014). Resting cysts (RCs) have different appearance, which can misleading in quantitative and qualitative studies. Compared to *A. pseudogonyaulax* cysts (40 to 50 µm in diameter) described by Bravo et al. (2006), mean cyst diameter of APBZ12 was 62.5 µm (SD ± 3.65). Most of *A. pseudogonyaulax* RCs encountered in this study were paratabulated, whereas others showed no tabulation at all or very fine tabulation hardly distinguished under photonic microscope.

The misidentification of *Alexandrium hiranoi* blooming on Jogashima, Japan (Murakami et al., 1988) as *Alexandrium pseudogonyaulax* (Kita et al., 1985) producing GDA caused a

taxonomic confusion about *A. pseudogonyaulax* toxicity. Kita and Fukuyo (1988) renamed formerly the dinoflagellate species producing GDA in Jogashima as *A. hiranoi*. However, unfortunately the confusion remained in many works dealing with GDA toxin (Mizuno et al., 1998; Matsunaga et al., 1999; Abe et al., 2002; Takeda et al., 2008).

To our knowledge, no study was conducted on well characterized strains of *A. pseudogonyaulax* to highlight any potential toxicity. The present work showed unambiguously using HPLC-MS, that *A. pseudogonyaulax* (APBZ12) produces GDA, the same toxin produced by *A. monilatum* from the Gulf Coast of the United States (Hsia et al., 2006) as well by *A. hiranoi* isolated from a Japanese rock pool (Murakami et al., 1988).

This toxin could be responsible of harmful effects on human health and aquatic fauna. Mizuno et al. (1998) reported that GDA affects human cell brain functioning causing morphological changes in astrocytomas cells by increasing the filamentous actin content. Also, GDA was reported to inhibit angiogenesis (regeneration of vessel) and that this was mediated at least in part through the inhibition of actin reorganization (Abe et al., 2002). During *A. monilatum* bloom in the lower York River, significant mortality of Veined rapa whelks (*Rapana venosa*, gastropods) was observed and GDA concentration was around 0.77–8.77 mg toxin g^{-1} of whelk tissue (Harding et al., 2009). Massive fish mortalities reported for over than 100 years in the Offats Bayou near Galveston, Texas (USA) have been associated to the GDA producer *A. monilatum* (Gates and Wilson, 1960, Hsia et al., 2006). This mortality was probably due to haemolytic activity of the produced polyether macrolide (Clemons et al., 1980; Bass et al., 1983).

At our knowledge, no blooms nor fish or mollusk mortalities have been yet associated with *A. pseudogonyaulax* from Bizerte. Monitoring programs on shellfish mortality is not yet implemented and aquaculture farms in Bizerte are rather uncommon. However, in view of the development of aquaculture activities and in regards of the significant cyst densities (Zmerli

Triki et al., 2014) in the sediment which are able to germinate and inoculate the water column, this lagoon is not immune to major development of this toxic dinoflagellate which could potentially cause the intoxication and the mortality of the wild fish and cultivated mollusks. Thus the existing monitoring program have to include *A. pseudogonyaulax* in HAB species list and GDA analyzes have to be performed when this dinoflagellate bloomed in the concerned marine ecosystem.

5. CONCLUSION

We have demonstrated that *A. pseudogonyaulax* produces the toxic compound goniodomin A. This toxin is similar to that produced by *A. monilatum* and *A. hiranoi* developing in contrasting marine systems. We also showed that the mass extract of APBZ12 was highly bioactive which suggest a possible lethal effect on fishes *in situ* when algal cells are disrupted. Further studies have to focus on the ecology and population dynamic of this species to better understand the main environmental factors regulating its development *in situ*. Ecophysiological studies have to investigate the modulation of the production of GDA by the environmental factors such temperature, salinity and nutrients (organic and inorganic nitrogen and phosphate). Because of the demonstrated bioactivity of GDA from APBZ12, it will be interesting to investigate the effect of this toxin on the major component of eleutheroschisis where a vegetative cell sheds its theca when settling on surface sediment, thereafter the resulting temporary cyst divides the marine ecosystem as fishes, mollusks and zooplankton and to highlight the transfer of this potent toxin to high trophic levels.

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494 **FIGURE CAPTIONS**

495

496 Fig. 1. Map of the Bizerte lagoon showing connections with Ichkeul lake and Mediterranean
497 sea.

498

499 Fig. 2. *Alexandrium pseudogonyaulax* resting cysts (RCs) morphotypes isolated from recent
500 sediments of Bizerte lagoon and life history stages: flattened side of *A .pseudogonyaulax* RCs
501 (A, D), paratabulated cyst (B-F); thin pellicule mucus in RC (narrow) (E), vesiculos content
502 of *A. pseudogonyaulax* RCs (F,G); granular content of *A. pseudogonyaulax* (H); RCs with two
503 (J, K) or four (I) daughter cells inside the cyst; paratabulated empty cyst (L); vegetative cell
504 (M); gamete (N); planozygote (O); laboratory produced resting cysts (P). Scale bar (10 µm).

505

506 Fig. 3. *Alexandrium pseudogonyaulax* APBZ12 designed thecal plates (1' to 4' and 1''to 6")
507 of the epitheca, Vp means ventral pore.

508

509 Fig. 4. Maximum likelihood phylogenetic tree of *Alexandrium pseudogonyaulax* inferred
510 from partial LSU rDNA (matrix of 42 taxa and 657 positions). The tree was routed with
511 *Goniodoma polyedricum*. Filled black circles indicate full statistical support of branches
512 (100/1.00). Values associated with branches correspond to bootstrap support and posterior
513 probabilities (ML/BI).

514

515 Fig. 5. (A) MALDI-TOF of toxic extract; 791.23 indicates mass of extract in positive ion
516 mode. (B) ESI-MS of toxic extract; 791.2 indicates mass of extract in positive ion mode

517

518 Fig. 6. Chemical structure of goniodomin A (reproduced from Hsia et al., 2006)

519 **Fig. 1**

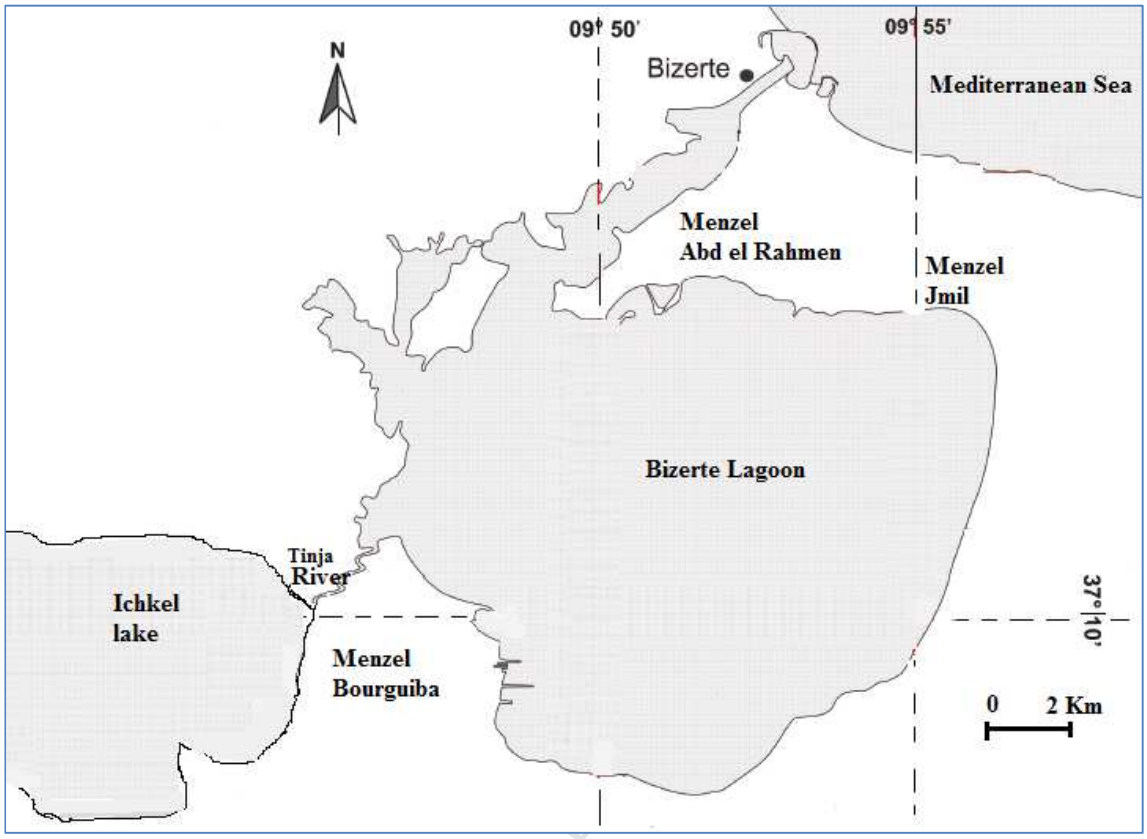

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Fig. 2

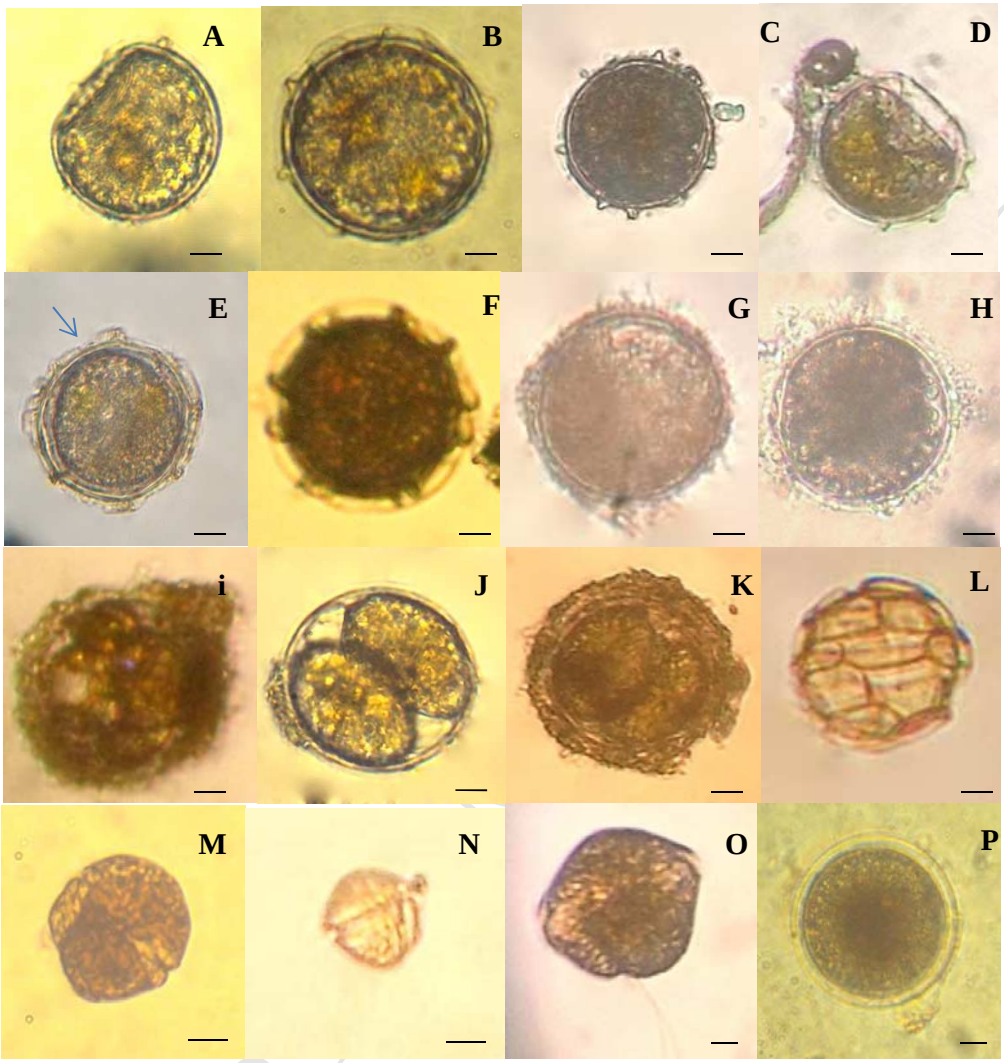
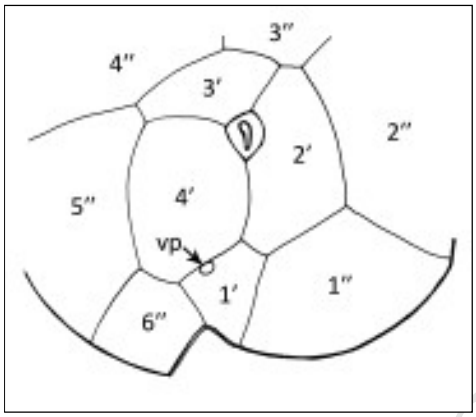
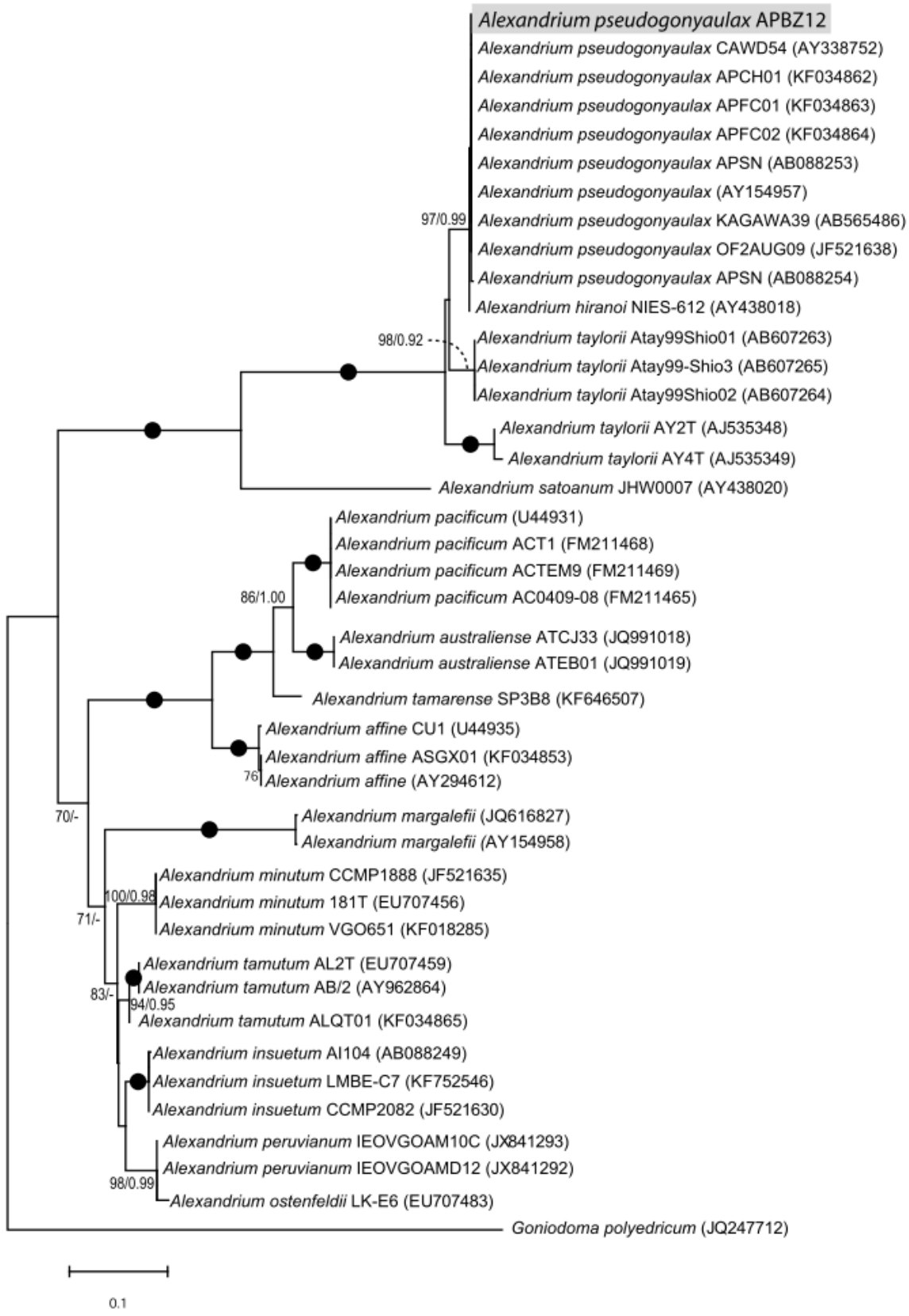


Fig. 3



546 **Fig. 4**


Alexandrium pseudogonyaulax APBZ12
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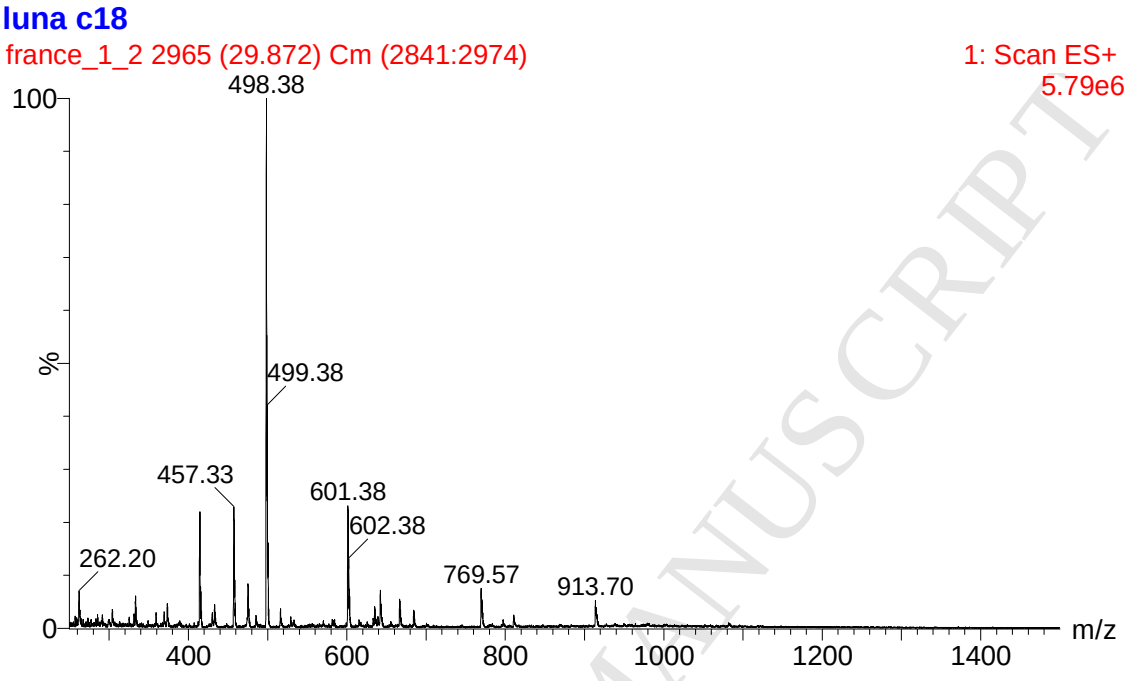
Alexandrium pseudogonyaulax APCH01 (KF034862)
Alexandrium pseudogonyaulax APFC01 (KF034863)
Alexandrium pseudogonyaulax APFC02 (KF034864)
Alexandrium pseudogonyaulax APSN (AB088253)
Alexandrium pseudogonyaulax (AY154957)
97/0.99
Alexandrium pseudogonyaulax KAGAWA39 (AB565486)
Alexandrium pseudogonyaulax OF2AUG09 (JF521638)
Alexandrium pseudogonyaulax APSN (AB088254)
Alexandrium hiranoi NIES-612 (AY438018)
98/0.92
Alexandrium taylorii Atay99Shio01 (AB607263)
Alexandrium taylorii Atay99-Shio3 (AB607265)
Alexandrium taylorii Atay99Shio02 (AB607264)
Alexandrium taylorii AY2T (AJ535348)
Alexandrium taylorii AY4T (AJ535349)
Alexandrium satoanum JHW0007 (AY438020)
Alexandrium pacificum (U44931)
Alexandrium pacificum ACT1 (FM211468)
Alexandrium pacificum ACTEM9 (FM211469)
86/1.00
Alexandrium pacificum AC0409-08 (FM211465)
Alexandrium australiense ATCJ33 (JQ991018)
Alexandrium australiense ATEB01 (JQ991019)
Alexandrium tamarense SP3B8 (KF646507)
Alexandrium affine CU1 (U44935)
Alexandrium affine ASGX01 (KF034853)
76
Alexandrium affine (AY294612)
70/-
Alexandrium margalefii (JQ616827)
Alexandrium margalefii (AY154958)
Alexandrium minutum CCMP1888 (JF521635)
100/0.98
Alexandrium minutum 181T (EU707456)
71/-
Alexandrium minutum VGO651 (KF018285)
Alexandrium tamutum AL2T (EU707459)
83/-
Alexandrium tamutum AB/2 (AY962864)
94/0.95
Alexandrium tamutum ALQT01 (KF034865)
Alexandrium insuetum AI104 (AB088249)
Alexandrium insuetum LMBE-C7 (KF752546)
Alexandrium insuetum CCMP2082 (JF521630)
Alexandrium peruvianum IEOVGOAM10C (JX841293)
Alexandrium peruvianum IEOVGOAMD12 (JX841292)
98/0.99
Alexandrium ostenfeldii LK-E6 (EU707483)
Goniodoma polyedricum (JQ247712)
0.1

547

548 **Fig. 5A**

549



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553 **Fig. 5B**

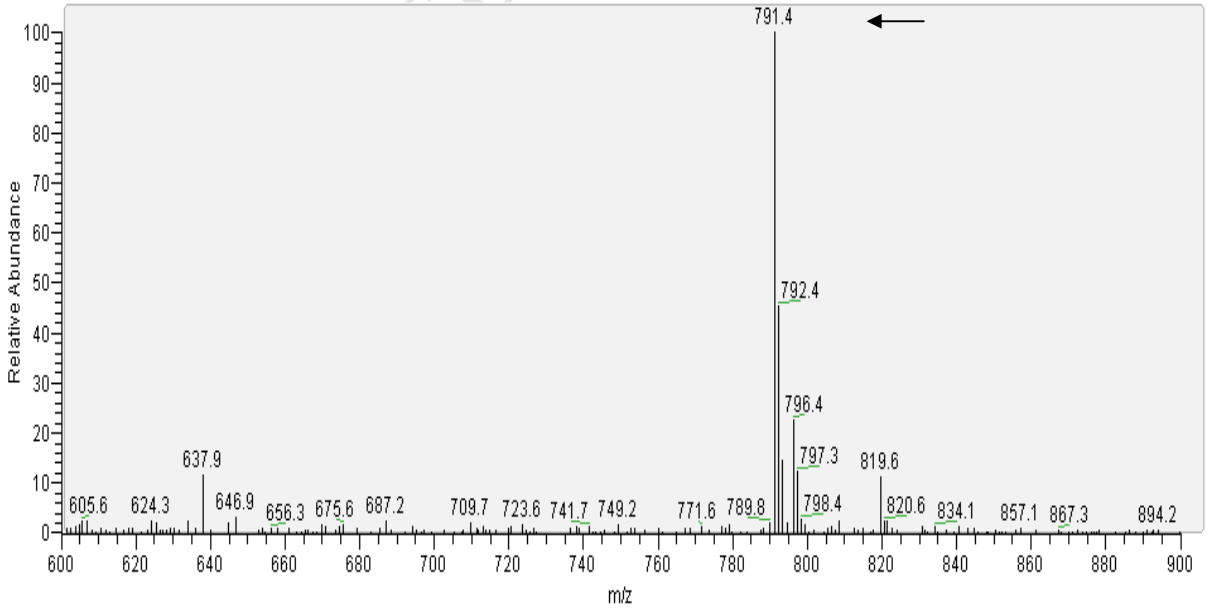

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Fig. 6