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Morphology, toxin composition and LSU rDNA phylogeny of *Alexandrium minutum* (Dinophyceae) from Denmark, with some morphological observations on other European strains

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Abstract

The morphology of *Alexandrium minutum* Halim from Denmark was studied and compared to the morphology of material from Portugal, Spain, France and Ireland. Strains from Denmark and the French coast of the English Channel differed from the typical *minutum* morphotype by the absence of a ventral pore. Cells without a pore also dominated field material from Ireland but a small fraction (6%) did have a pore. Many cells had a heavily areolated theca. In the exponential growth phase, the PSP-toxin profile of the Danish strain of *A. minutum* was dominated by C1 and C2 (up to 70%), whereas GTX2 and 3 made up more than 17%, and STX almost 13%. Cells entering the stationary phase contained 30% STX with a concomitant decrease of the other toxins. Partial large subunit rDNA sequences (664 bp) confirmed that the Danish *A. minutum* strain clusters together with other European strains of this species, and a strain from Australia. However, sequencing of this part of the gene did not resolve intraspecific relationships and could not differentiate populations with or without pore and/ or different toxin signatures. A strain from New Zealand had a remarkably high sequence divergence (up to 6%) compared to the other strains of *A. minutum* and its identity should be further investigated. A distribution map of *A. minutum* has been compiled and it is suggested that *A. minutum* and *A. angustitabulatum* Taylor are conspecific.

Keywords: *Alexandrium minutum*; Biogeography; Distribution; LSU rDNA; Morphology; Phylogeny; PSP toxins; SEM; Taxonomy

1. Introduction

The dinoflagellate genus *Alexandrium* Halim comprises some of the most potentially toxic algae. So far, 28 species have been described, 11 of which have been confirmed toxic (Balech, 1995; Taylor et al., 1995; MacKenzie and Todd, 2002; Moestrup et al., 2002). The toxins consist of a suite of more than 20 tetrahydropurin derivatives collectively known as paralytic shellfish poisoning (PSP) toxins, referring to their accumulation in shellfish and mode of action (e.g. Kao, 1993; Kodama, 2000). Species of *Alexandrium* are widely distributed (e.g. Scholin et al., 1994; Scholin et al., 1995) and their presence may be devastating for human health and shellfish industry. This has led to intensive research of the genus, e.g. taxonomy, toxin chemistry, lifecycles, ecology and biogeography (e.g.

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Fukuyo, 1985; Balech, 1995; Scholin et al., 1995; Anderson, 1998; Cembella, 1998; Shimizu, 2000, and references therein).

Many *Alexandrium* species have had a very confusing taxonomic history and have been classified in *Gessnerium* Halim, *Goniodoma* Stein, *Gonyaulax* Diesing, *Protogonyaulax* Taylor and *Pyrodinium* Plate (see Balech, 1995 for a historic treatment). The confusion at the generic level was resolved by Balech's (1989) redescription of the type species, *Alexandrium minutum*, and emendation of the genus. At present, the classification of species within *Alexandrium* is primarily based on details of the thecal plate pattern. Generic characteristics include the thecal plate formula Po, 4′, 6″, 5‴, 2⁗, 6c and 9–10s. Species have relatively thin and smooth thecal plates except for *A. insuetum*, which is strongly reticulated, *A. balechii*, which has irregular crests or ridges, and to some extent *A. minutum*, which sometimes has a reticulated hypotheca (Balech, 1995; Montresor et al., 1990). Features used for species separation are primarily minute details of the plate pattern such as shape and size of the 1′, 6″, s.a. and s.p. plates, details of the Po plate and the presence or absence of a ventral pore, typically situated at the margins between the 1′ and 4′ plates. Several molecular studies using LSU or SSU rDNA nucleotide sequences have shown *Alexandrium* to be monophyletic (Saunders et al., 1997; Daugbjerg et al., 2000). However, molecular studies have also challenged the species concept within the genus. Hence, species of the *Alexandrium tamarense* complex group according to their geographical origin rather than to their morphotype (e.g. Scholin et al., 1994, 1995).

In June 1992, a bloom of *Alexandrium* sp. (134,000 cells l^{-1}) took place at Korsør Nor, a small Danish embayment on west Sealand (Brix-Madsen, personal communication). The embayment is a highly eutrophic locality that received treated sewage until 1995 (Anonymous, 1995). The bloom forming *Alexandrium* species resembled *A. minutum*, but lacked a ventral pore and was therefore tentatively identified as *Alexandrium* cf. *angustitabulatum*, which lacks a ventral pore (Balech, 1995). A lugol-fixed sample was sent to Balech for a more precise identification. He rejected it as being *A. angustitabulatum* and based his judgement on the shape of the first apical plate. However, he agreed that the plate pattern was very similar to *A. minutum*, except for the lack of a ventral pore. No conclusions were reached with regard to the identity of this species (Balech, personal communication). In July 1997, another bloom occurred at the same location, the concentration this time reaching 222,000 cells l^{-1} (Brix-Madsen, personal communication), indicating that maximum occurrence was a summer phenomenon. In June 2001, live samples were collected and cultures established for further analyses. The present study provides details of the plate pattern, toxin composition and sequence analysis of partial LSU rDNA. The DNA nucleotide sequence data reveal that the Danish *Alexandrium* isolate is similar to *A. minutum* strains from other geographical locations. Additional preserved field samples and cultures of other European *A. minutum* populations have been included for comparative morphological analyses.

2. Material and methods

2.1. Cultures and samples

Clonal cultures were established from a net sample collected at Korsør Nor (Denmark), 26 June 2001. They were grown either in TL-medium (http://www.sccap.bot.ku.dk) at 16 PSU, 20 °C and a photon flux of ca. 100 μmol m^{-2} s^{-1} or L1-medium (Guillard and Hargraves, 1993) at 34 PSU, 15 °C and a photon flux of ca. 25 μmol m^{-2} s^{-1}. The latter growth conditions were used for toxin analysis. The strain primarily used was designated GHmin04. Lugol-fixed material from the 1997 bloom was also included in the morphological analysis. Additionally, formalin-fixed strains (3.7% final concentration) were: Al1V from Galicia, Spain, A18 from Portugal (formerly known as *A. lusitanicum*), a strain from Brittany, France designated Nantes, and a Lugol-fixed net sample collected about 30 mile off the Connemara coast, western Ireland, 27 July 2001.

2.2. Light microscopy

Cells stained with CalcoFlour White (Fritz and Triemer, 1985) were examined by Olympus BHS-2, BX 60 or Provis AX70 microscopes fitted with a filter arrangement for violet excitation (400–410 nm). Photographs were taken by a PM10-ADS

microphotosystem using Technical Pan film (Eastman Kodak Company) or by Axiocam (Zeiss) or DP-10 (Olympus) digital cameras.

2.3. Scanning electron microscopy (SEM)

For SEM, cells were fixed in either formalin or Lugols solution as outlined in Section 2.1, dehydrated in a graded ethanol series, critical point dried and coated with platinum. The microscope used was a Jeol JSM-6335F operated at 5 kV.

2.4. Toxin analysis

HPLC analyses of PSP toxins were performed on 10- and 13-day-old cultures, corresponding to late exponential and early stationary growth phases, respectively. The cultures were filtered onto GF/C filters, extracted twice with 0.05 M acetic acid, homogenized with an ultrasonic probe and analyzed as described in Franco and Fernandez Vila (1993). Appropriate standards were used for verification and quantification of STX, dcSTX, and GTX1–4. However, for the potential presence of GTX5 and 6 and C-toxins, samples were hydrolyzed to provide the corresponding STX, neoSTX and GTX1–4 toxins (Franco and Fernandez Vila, 1993).

2.5. DNA extraction, PCR amplification and sequencing of partial LSU rDNA

Approximately 10 ml of an exponentially growing culture of *A. minutum* (GHmin04) were concentrated by centrifugation (2500 rpm for 10 min). The pellet was kept frozen at −18 °C until extraction of total genomic DNA. With a few modifications, the CTAB extraction of DNA followed the procedure described by Doyle and Doyle (1987). Cells were incubated in 500 μl preheated 2 × CTAB isolation buffer (2% hexadecyltrimethylammonium bromide) at 60 °C. Following incubation for 1 h, total genomic DNA was extracted using 500 μl 24:1 chloroform:isoamyl alcohol and precipitated using 1 ml 96% ethanol and 50 μl 3 M sodium acetate. Double-stranded DNA was amplified in 50 μl of a solution containing 5 μl 10× Taq buffer (67 mM Tris–HCl, pH 8.5, 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$ and 10 mM β-mercaptoethanol), 20 μl 0.5 μM dNTP mix, 5 μl 10 μM of each primer, 5 μl 100 mM tetramethylammonium chloride (TMA) and 1 U Taq polymerase (Amersham, UK). The amplification primers used were D1R-F (Scholin et al., 1994) and 28–1483R (Daugbjerg et al., 2000). PCR conditions were one initial cycle of denaturation at 94 °C for 3 min followed by 35 cycles each consisting of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min. The temperature profile was completed by a final extension step at 72 °C for 6 min. The DNA fragments were checked on 2% Nusieve gels containing EtBr, and visualized by ultraviolet illumination. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany) following the recommendations of the manufacturer. Twenty-five nanograms of purified PCR product were used in 20 μl sequencing reactions. Nucleotide sequences were determined using the dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, California) as recommended by the manufacturer. Sequencing reactions were run on an ABI PRISM 377 DNA sequencer (Perkin-Elmer). In order to obtain sequences in both directions, we used two amplification primers in addition to internal primers D3A, D2C and D3B (see Scholin et al., 1994; Nunn et al., 1996 for primer sequences).

2.6. Alignment, outgroup and phylogenetic analyses

The partial LSU rDNA sequence was aligned with 32 other taxa of *Alexandrium* (in total 12 species), all retrieved from GenBank (Table 1). For most *Alexandrium* species in GenBank, only domains D1 and D2 have been determined. Hence, our alignment included only the first two domains of the LSU rDNA, in total 664 bp. A recent study by Daugbjerg et al. (2000) showed *Fragilidium subglobosum* as sister group to *Alexandrium*. Hence, we used this taxon to polarize the in-group comprising species of *Alexandrium*. The aligned nucleotides were subjected to parsimony, maximum likelihood and neighbour-joining analyses. All phylogenetic analyses were performed using PAUP*, version 4.0b10 (Swofford, 2003). Parsimony analyses were done using the heuristic search option with random additions (1000 replicates) of sequences and a branch-swapping algorithm (TBR). Gaps were treated as missing data and characters were treated as multistate and unordered. In maximum likelihood

Table 1
List of *Alexandrium* species included in the phylogenetic analyses

Taxon	Strain	Geographic region	GenBank accession number
A. affine (Inoue and Fukuyo) Balech	X21	France, Bay of Concarneau	AF318229
A. affine	CU1	Thailand, Gulf of Thailand	U44935
A. affine	Pa4V	Spain	L38630
A. affine	–	Vietnam	AY294612
A. catenella (Wheedon and Kofoid) Balech	HK1989	Hong Kong	AF118546
A. catenella	ATT98	France	AF318219
A. catenella	GHope1	South Korea, ballast water	–
A. catenella	ACBOPNZ	New Zealand	AF019408
A. catenella	A3	US, west coast	AF200667
A. concavum (Gaarder) Balech	CAWD52	New Zealand, North Island	AF032348
A. excavatum (=*A. tamarense*) (Braarud) Balech and Tangen	GE1V	–	L38632
A. fundyense Balech	AFNFA3.2	Canada, east coast (Newfoundland)	U44928
A. insuetum Balech	X6	France, Corsica	AF318233
A. lusitanicum (=*A. minutum*) Balech	A18	Portugal	L38623
A. margalefii Balech	–	Denmark, from cyst	AY154957
A. minutum Halim	AMAD06	Australia, Port River, S.A.	U44936
A. minutum	GHmin04	Denmark, Korsør Nor	AY294613
A. minutum	AM89BM	France, Morlaix	AF318221
A. minutum	91/2	France, Antifer Harbour	AF318262
A. minutum	95/1	France, Bay of Concarneau	AF318263
A. minutum	X13	France, Bay of Toulon	AF318231
A. minutum	X20	France, The Rance	AF318232
A. minutum	ANAKOHA	New Zealand, Anakoha Bay	AF033532
A. minutum	Al1V	Spain, Galicia	L38626
A. ostenfeldii (Paulsen) Balech and Tangen	NZost	New Zealand, Wellington	AF033533
A. pseudogonyaulax (Biecheler) Horiguchi ex Yuki and Fukuyo	–	Denmark, from cyst	AY154958
A. tamarense (Lebour) Balech	OF041	Japan, Ofunato Bay	U44929
A. tamarense	PW 06	US, west coast	U44927
A. tamarense	K-0055	Faroe Islands, Tjaldavik	AF200668
A. tamarense	Pgt183 (=PHY1)	United Kingdom, Plymouth	U44930
A. tamarense	Plymouth	–	AF033534
A. tamarense	ATBB01	Tasmania, Bell Bay	U44933
Fragilidium subglobosum[a] (von Stosch) Loeblich	–	–	AF033868

Unavailable data is indicated by '–'.
[a] Outgroup taxon.

analyses, Modeltest (version 3.06) was used to find the optimal model using a 0.05 level of significance (Posada and Crandall, 1998). The model that best fitted the data was TrN+G (Rodriguez et al., 1990) with estimated base frequencies and included parameters for rate heterogeneity between sites: the proportion of sites assumed to be invariable was none and a gamma distribution with four rate categories was invoked on the remaining sites. These parameters were added prior to maximum likelihood runs in PAUP* and heuristic searches with 10 random additions were performed. Exact parameters were estimated from consecutive heuristic searches (also with 10 random additions) and reoptimized until the parameter values converged. Optimal parameters were a substitution matrix: (a b a a e a) = (1, 2.7284, 1, 1, 5.4108, 1), the proportion of invariable sites was 0 and the shape of the gamma distribution, $\alpha = 0.5606$. The same model as in maximum likelihood was used to compute dissimilarity values. The distance matrix was used to build a tree with the neighbor-joining method. Bootstrap values were conducted to examine the robustness

of clades using 'fast' stepwise addition (100 replicates in maximum likelihood and 100,000 replicates in parsimony with reweighing according to the rescaled consistency index over an interval of 1–1000). In neighbor-joining bootstrap analyses 1000 replicates were performed.

3. Results

3.1. Morphology

Cells from Denmark were somewhat ovoid in ventral view and measured 26.5 ± 2.4 μm in length and

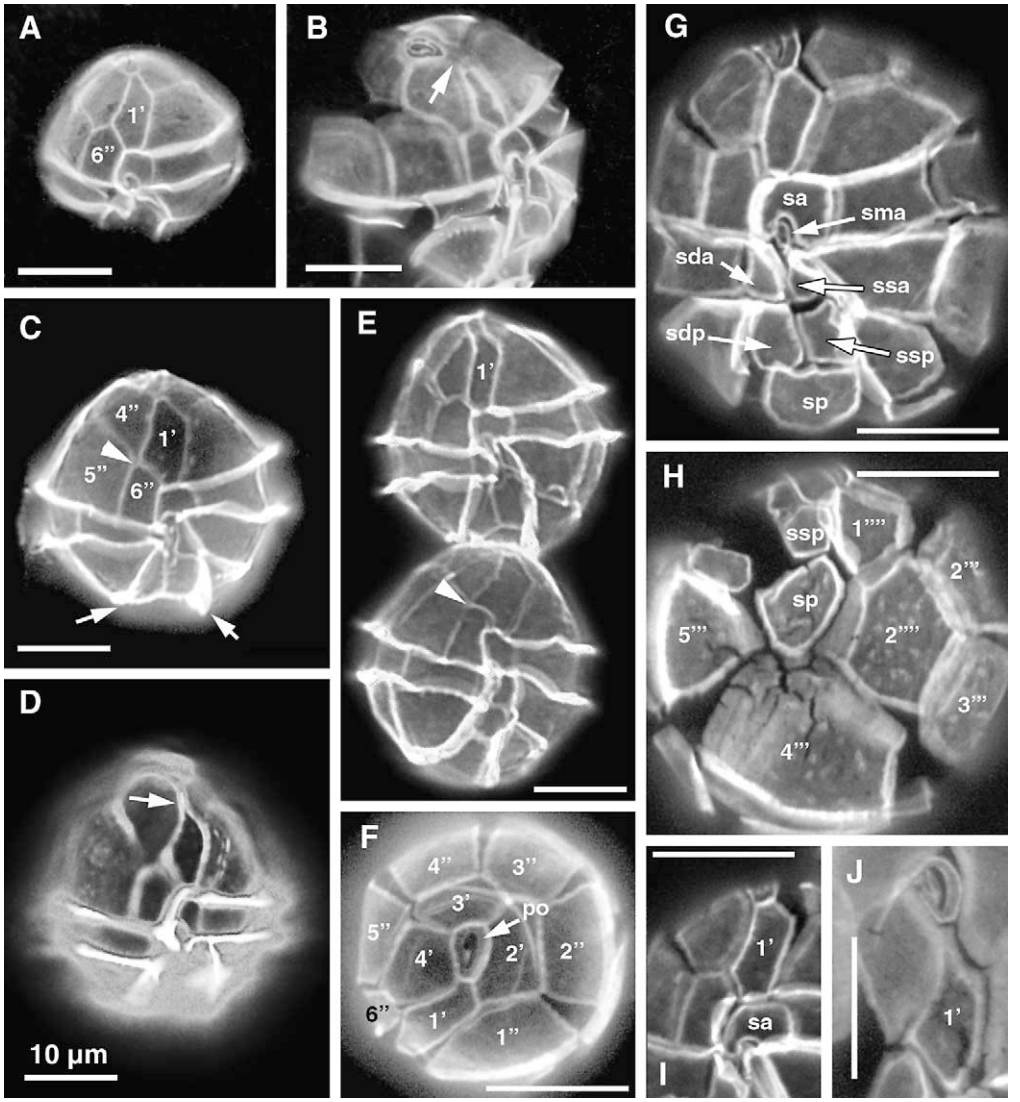

Fig. 1. Fluorescence microscopy of CalcoFlour White-stained *Alexandrium minutum* specimens from Denmark (A and B: field material from the 1992 bloom; C–J: strain GHmin04). (A) Ventral view of a specimen with relatively narrow connection between the Po and 1′ plate. (B) Specimen with well-developed intercalary bands. The 1′ plate looks as if displaced from the Po plate (arrow). (C) The sulcal lists (arrows) and the very reduced 4″–6″ suture (arrowhead). (D) The 1′ plate may also appear disconnected from the Po plate in cultured material (arrow). (E) Newly divided cells still attached to each other. Notice the different shape of the 1′ plate, with almost parallel sides in the upper cell, and the extremely reduced 4″–6″ suture in the lower cell (arrowhead). (F) Epithecal plate pattern. (G) Sulcal plates. (H) Hypothecal plate pattern. (I and J) Different shapes of the 1′ plate. Note also the relatively long s.a. plate in (I).

24.8 ± 2.2 μm in width ($n = 30$). No morphological differences were found between field material from the bloom in 1992 and the cultures established in 2001.

The plate tabulation was typical for *Alexandrium*. The Po plate had a distinct comma (Fig. 1B, F and J) and scattered marginal pores, but these were difficult to see without the use of SEM (not shown). The first apical plate (1′) displayed considerable variation in shape. In some specimens, the plate had a more or less concave right anterior margin bordering the 4′ plate, whereas in others it was rather narrow, with straight and almost parallel right and left margins (Fig. 1A, C–E, G, I and J). This variation also occurred within newly divided cells still attached to each other (Fig. 1E). In senescent cells, the intercalary bands of the 2′ and 4′ apical plates often joined, and the 1′ apical plate appeared to be disconnected from the Po plate (Fig. 1B and D). However, it was connected to the Po plate by a more or less narrow extension (Fig. 1J). The anterior tip of the 1′ plate sometimes appeared divided, indicating the presence of a small canal plate. However, this was caused by a transverse ridge of the thecal plate as seen in SEM (Figs. 1E and 2A–C). No trace of a ventral pore was present in the several hundreds of specimens examined. The 6″ plate was narrow with a length:width ratio of 1.8:2 (Fig. 1A–G and I). Considerable variation was observed in the length of the suture between 1′ and 6″ (e.g. Fig. 1A and G) and the suture between 4′ and 6″ (Fig. 1A and C). In some cells, the 4′–6″ suture was reduced to the extent that 1′, 4′, 5″ and 6″ joined in a single point (Fig. 1E). This has been termed a quadruple junction and is rather unusual (see Evitt, 1985). The anterior sulcal plate (s.a.) usually had a more or less straight anterior margin and a length:width ratio close to one (Fig. 1A and G). However, in some specimens, the width of s.a. was almost twice its length (Fig. 1I). The s.s.p. plate varied from almost isodiametrical to considerably longer than width (Fig. 1G and H). The posterior sulcal plate (s.p.) was short, about 1.5× wider than the length (Fig. 1G and H). Both the right and left border of the sulcus had distinct sulcal lists made by extensions of the 5‴, 1‴ and 1⁗ plates (Figs. 1C and 3A). Thecal plates were always smooth with scattered pores (Figs. 1H and 3A).

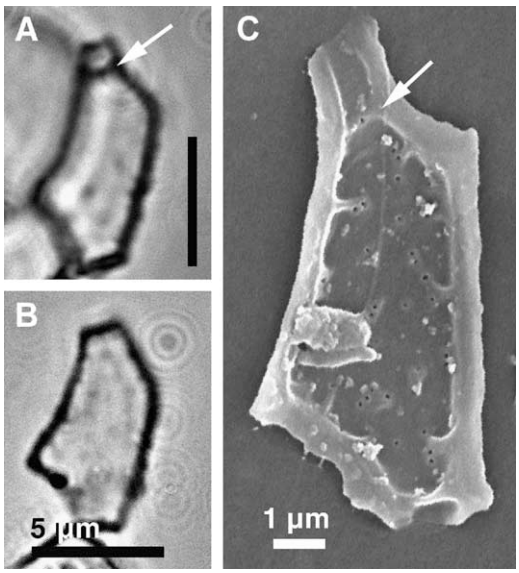

Fig. 2. (A) Light micrograph of the 1′ plate with a transversal ridge in the apical part (arrow). (B) 1′ plate without the ridge. (C) SEM micrograph of the 1′ plate confirming the presence of a small ridge on the plate (arrow).

The strain from Brittany (France) showed considerable resemblance to the material from Denmark by lacking a ventral pore and having a smooth theca, though some specimens had slightly reticulated thecal plates (Fig. 3B). In contrast, the strains from Spain (Al1V) and Portugal (A18) had a distinct ventral pore made by complementary small depressions or notches of the left and right margins of the 4′ and 1′ plates, respectively (Fig. 3C and D). Many specimens of the Spanish strain had more or less reticulated thecal plates, while this was less pronounced in the Portuguese strain (Fig. 3C and D).

In the field sample from western Ireland, the majority of cells lacked a ventral pore (Fig. 4A, B and D). However, about 6% ($n = 150$) had a ventral pore, but this was less distinct than in the Spanish and Portuguese strains (Fig. 4C). Many of the Irish cells had very thick and areolated plates (Fig. 4D) and generally the plates also appeared thicker in smooth-walled cells, as revealed by the excellent preservation of the cells for SEM compared to cultured material. In some specimens, half the theca was heavily armoured and the other half smooth (Fig. 4A 2and B), a clear demonstration of the desmoschitic division mode where the daughter cells inherit half the parent theca and form the other half de novo (e.g. Pfiester and Anderson, 1987). Extended growth margins of the 2′ and 4′ again gave the impression of a 1′ plate disconnected from the Po plate (Fig. 4B).

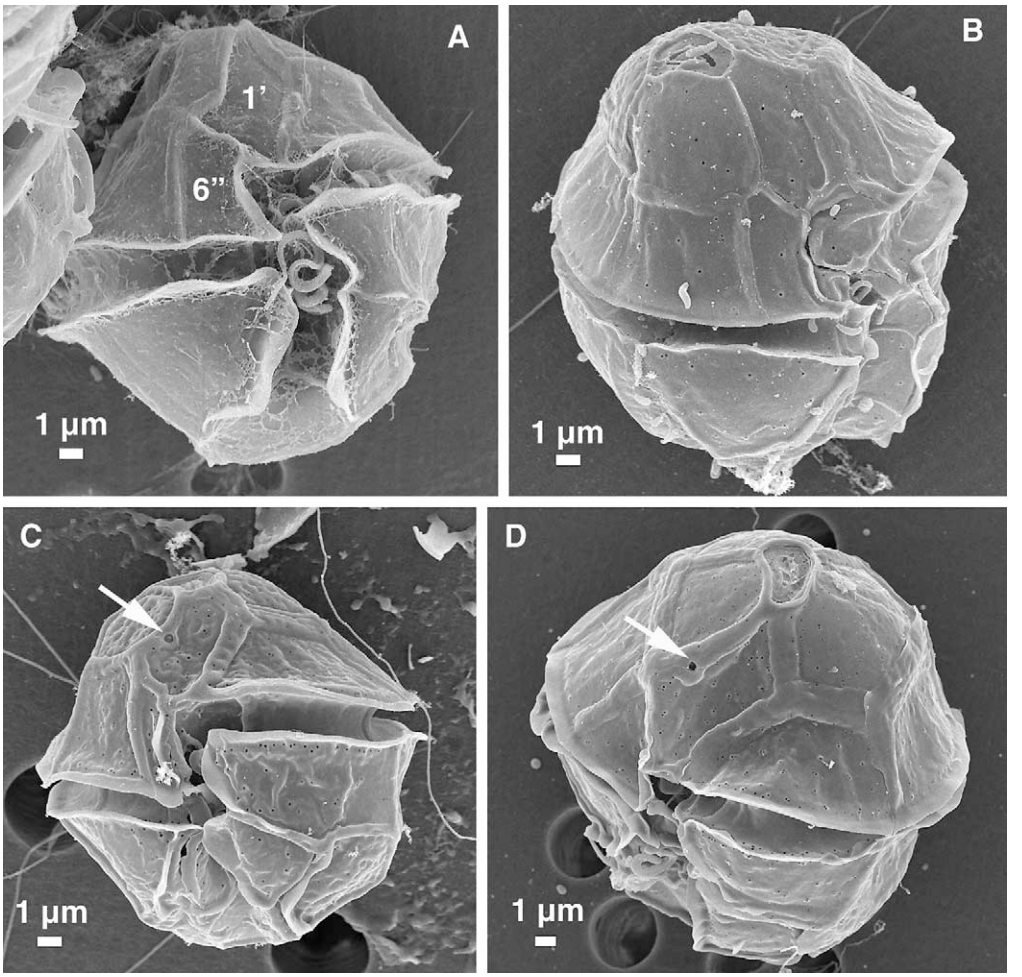

Fig. 3. SEM micrographs of *Alexandrium minutum*. (A) Strain GHmin04 from Denmark. (B) Strain Nantes from France. (C) Strain Al1V from Spain, with ventral pore (arrow). (D) Strain A18 from Portugal, also showing a ventral pore (arrow).

3.2. Toxins

In late exponential growth phase, the toxins of the Danish strain of *A. minutum* (day 10) were dominated by C1 and C2 toxins, which constituted about 70%. GTX2 and 3 made up ca. 17%, and STX almost 13% (Fig. 5, Table 2). In cells entering the stationary phase (day 13), the relative proportions of toxins had changed, with a significant increase of STX to about 30%, and concomitant decrease of the other toxins (Table 2). Traces of GTX5 were probably present, as a slight increase of the STX peak after hydrolysis of the sample was noted (Fig. 5).

3.3. Comparison of partial LSU rDNA sequences in Alexandrium species

The LSU rDNA sequence of the Danish strain GHmin04 was identical to strain AMAD06 from Australia, and strain AM89BM from the Atlantic coast

Table 2
Dominant toxins (mol%) of *Alexandrium minutum* (GHmin04)

Day	Cells ml^{-1}	C1	C2	GTX3	GTX2	STX
10	33875	31.22	38.67	8.87	8.40	12.84
13	32800	26.78	30.19	6.42	6.56	30.05

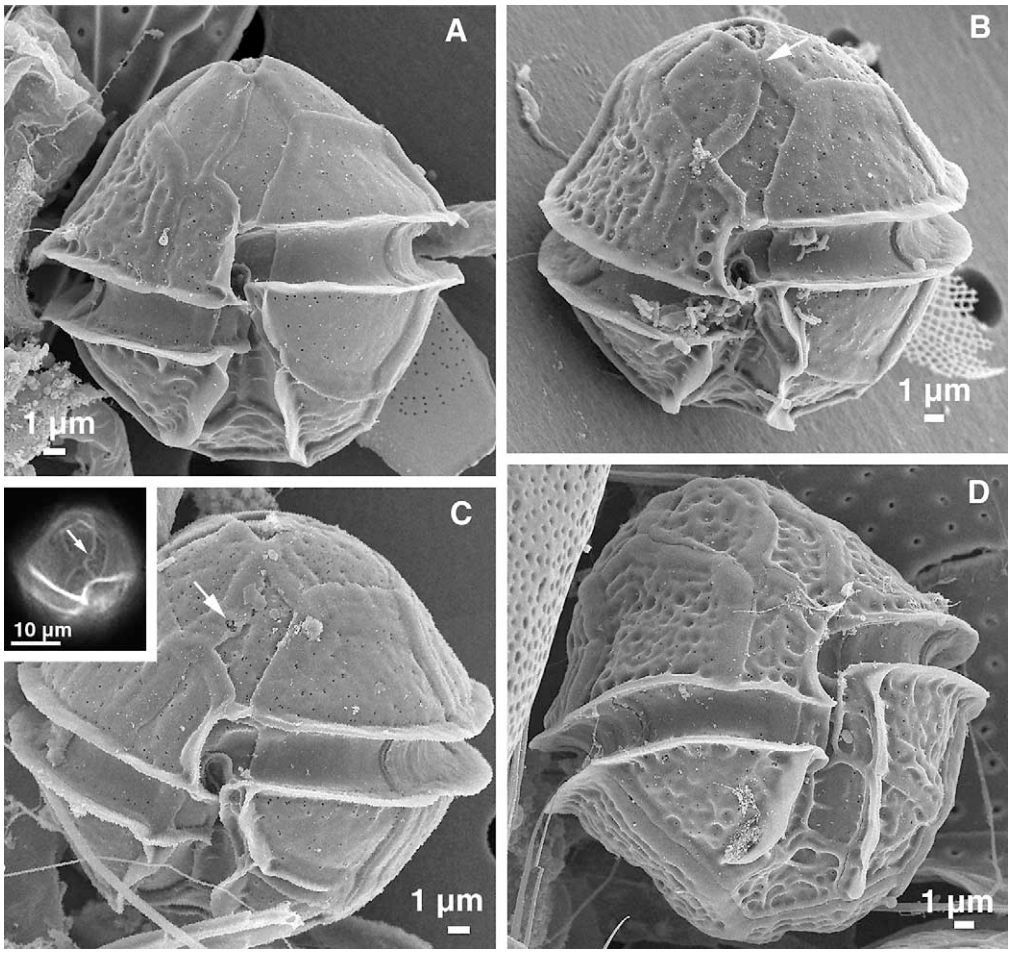

Fig. 4. SEM micrographs of *Alexandrium minutum* from western Ireland. (A) Cell with thick, ornamented parental plates in the right part of the cell and smooth plates in the left part. (B) The 1′ plate appears to be displaced from the Po plate (arrow) by the broad intercalary bands of plates 2′ and 4′. (C) Cell with ventral pore (arrow). Inset: fluorescence micrograph of CalcoFlour White-stained cell, the ventral pore is marked by an arrow. (D) Cell with thick areolated plates.

of France. The sequence divergence between these three strains and the other strains from the Atlantic coastal waters of France and Spain ranged from 0.16 to 1.7% (Table 3). The Spanish (Al1V) and Portuguese (A18) strains and the strains 91/2 and 95/1 from France were also identical. In *A. minutum* originating from the French coast of the English Channel, the sequence divergence was 0–1.3% when based on uncorrected nucleotide differences (Table 3). The Portuguese strain A18 has previously been referred to as *A. lusitanicum* (Scholin et al., 1994, as GtPort; Zardoya et al., 1995, as AL18A) but, like previous studies, the partial LSU rDNA revealed this isolate to be *A. minutum* (Table 3) On the other hand, the ANAKOHA strain from New Zealand, identified as *A. minutum* showed a nucleotide sequence divergence of 4.9–6% compared to all other strains of *A. minutum* (Table 3). The sequence divergence between the ANAKOHA strain and other species of *Alexandrium* (e.g. *A. insuetum* and *A. ostenfeldii*) was in the same range, 5–8% (Table 4).

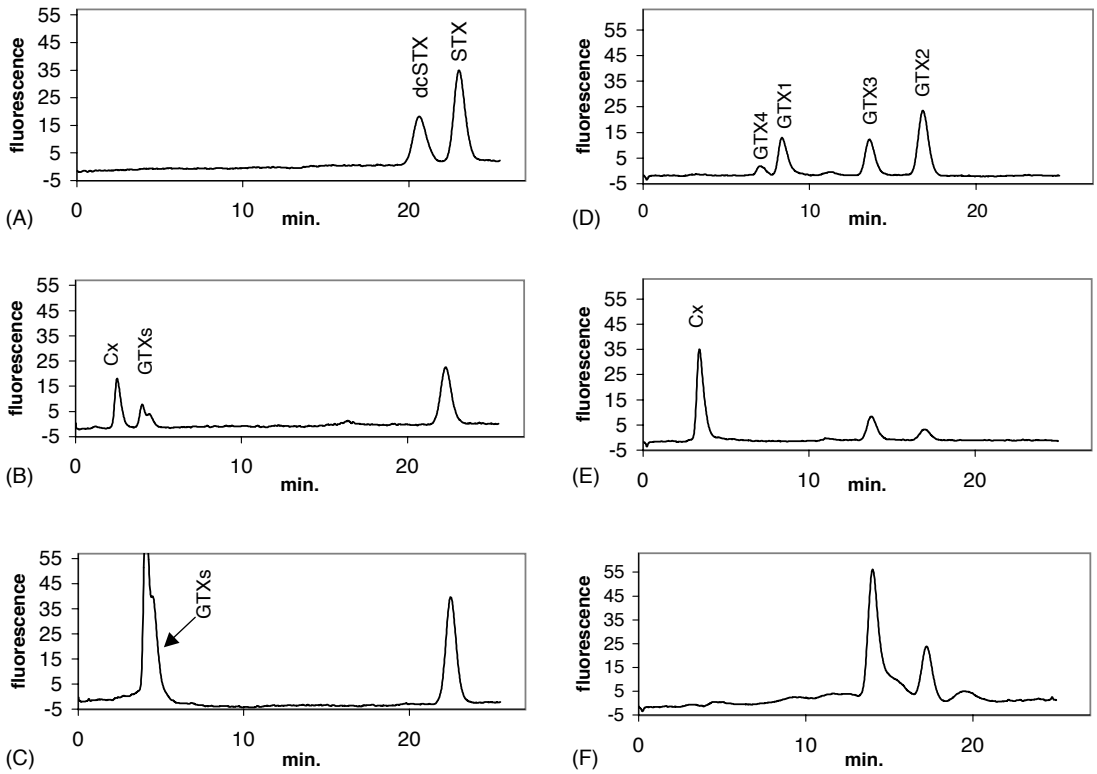

Fig. 5. HPLC chromatograms of PSP toxins of *Alexandrium minutum* (GH04 min). (A) Standards of dcSTX and STX. (B) Before hydrolysis. (C) After hydrolysis: the peak of Cx disappears, while the GTXs increase. The small increase of STX is due to traces of GTX5. (D) GTX standards. (E) Before hydrolysis. (F) After hydrolysis: the peak of Cx has disappeared and that of GTX3 and 2 have increased, indicating the presence of C1 and C2 toxins.

Table 3
Sequence divergence of species identified as *Alexandrium minutum* based on 664 bp of partial LSU rDNA

Taxa	AMAD06 Australia	GHmin04 Denmark	AM89BN France, Atlantic	91/2 France, Atlantic	95/1 France, Atlantic	X13 France, Mediterranean	X20 France, Atlantic	ANAKOHA New Zealand	Al1V Spain, Atlantic	A18 Portugal Atlantic
AMAD06	–	0.0	0.0	0.32	0.16	0.46	1.08	5.14	0.46	0.46
GHmin04	0.0	–	0.0	0.32	0.16	0.46	1.09	5.14	0.62	0.62
AM89BM	0.0	0.0	–	0.32	0.16	0.46	1.08	5.14	0.62	0.62
91/2	0.32	0.32	0.32	–	0.0	0.82	1.32	5.42	0.98	0.98
95/1	0.16	0.16	0.16	0.0	–	0.66	1.17	5.28	0.82	0.82
X13	0.46	0.46	0.46	0.81	0.66	–	1.56	5.64	1.09	1.09
X20	1.07	1.07	1.07	1.31	1.15	1.53	–	5.98	1.72	1.72
ANAKOHA	4.94	4.95	4.94	5.20	5.07	5.40	5.71	–	5.82	5.82
Al1V	0.46	0.61	0.61	0.97	0.81	1.08	1.70	5.57	–	0.0
A18	0.46	0.61	0.61	0.97	0.81	1.08	1.70	5.57	0.0	–

Divergence is given in percent (%). Uncorrected distances are given below the diagonal and distance estimates based on the Kimura-2-parameter model above the diagonal. See Table 1 for explanation of strain designations.

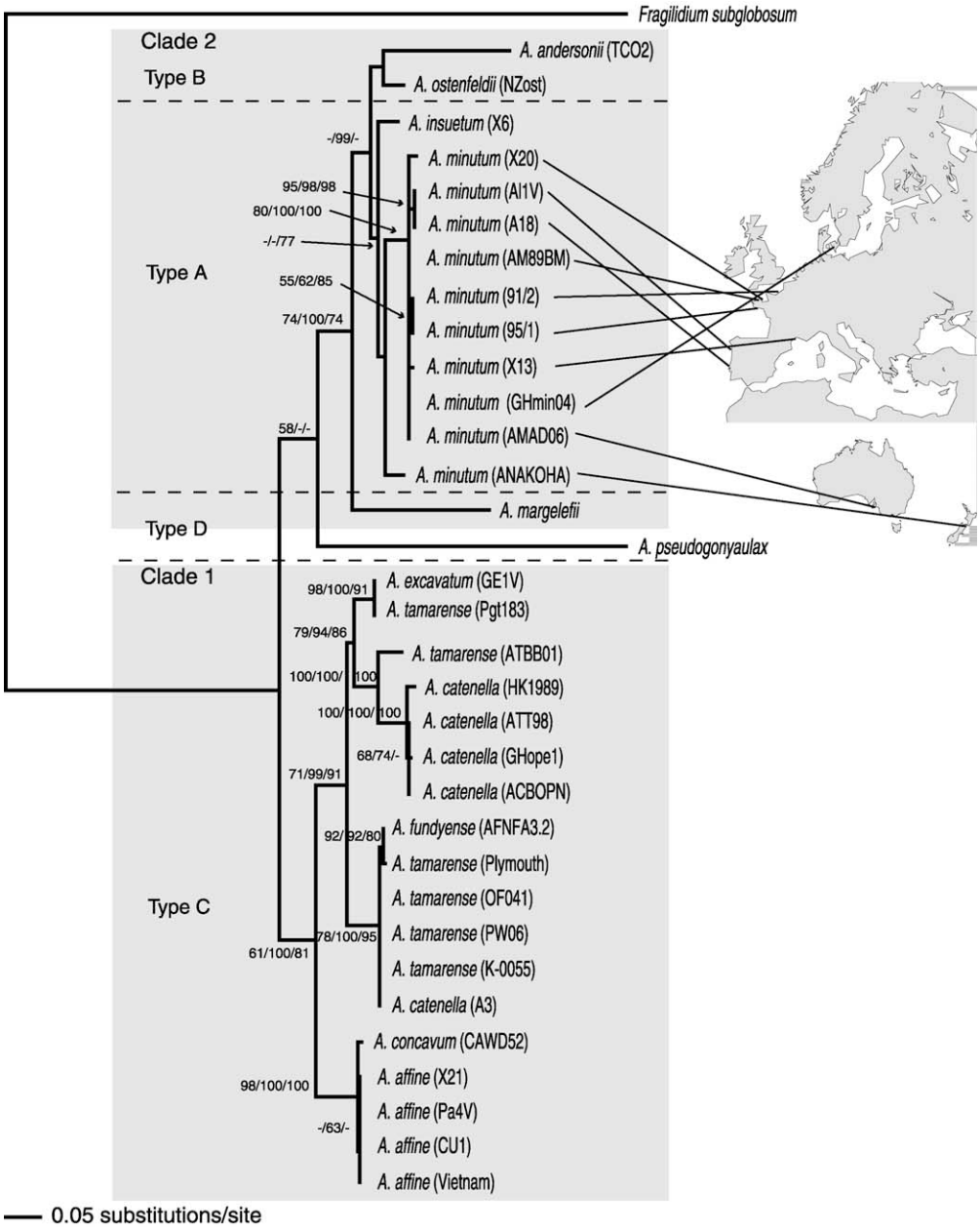

Fig. 6. Maximum likelihood tree based on nucleotide sequences of domains D1 and D2 of the LSU rDNA gene. The tree had a ln likelihood score of −4466.982 and was rooted using *Fragilidium subglobosum*, another dinoflagellate belonging to the Gonyaulacales. The alignment comprised 664 bp of which 248 were parsimony informative. The parsimony analysis produced 72 equally parsimonious trees, each with a length of 845 steps (not shown; consistency index = 0.731; retention index = 0.879). Numbers to the left of the clades are bootstrap values. The first numbers are from maximum likelihood analyses with 100 replicates using 'fast' stepwise addition. The second numbers are from parsimony analyses, with 100,000 replicates, also using "fast" stepwise addition but applying a re-weighted consistency index over an interval of 1–1000. The third numbers are bootstrap values from distance analyses with 1000 replicates. Types A–D refer to the shape of the posterior sulcal plate (s.p.) sensu Yoshida (personal communication).

Table 4
Sequence divergence in percent of four stains of *Alexandrium* based on 664 bp of partial LSU rDNA

Taxa	*A. minutum* GHmin04 Denmark	*A. minutum* ANAKOHA New Zealand	*A. ostenfeldii* NZost New Zealand	*A. insuetum* X6 Corsica
GHmin04	–	5.14	8.389	5.94
ANAKOHA	4.95	–	7.40	5.45
NZost	7.84	7.01	–	6.54
X6	5.69	5.24	6.22	–

Uncorrected distances are given below the diagonal and distance estimations based on Kimura-2-parameter model above the diagonal. See Table 1 for explanation of strain designations.

3.4. Phylogeny and population structure of Alexandriumspecies

A data matrix comprising 664 nucleotides of domains D1 and D2 of the LSU rDNA gene was compiled for 33 taxa (including 10 strains identified as *A. minutum*) in order to elucidate the phylogeny and population structure of isolates of *Alexandrium* species. The maximum likelihood inference illustrated in Fig. 6, and also parsimony and neighbor-joining analyses, revealed two major clades. Clade 1 comprised *A. concavum*, *Alexandrium affine* and the *tamarense* species complex sensu Scholin et al. (1995) comprising *A. tamarense* (including *excavatum*), *A. fundyense* and *A. catenella*. Clade 2 comprised *A. margalefii*, *A. ostenfeldii*, *A. andersonii*, *A. insuetum* and *A. minutum*. *Alexandrium pseudogonyaulax* formed a sister group to clade 2, although this relationship was only moderately supported in maximum likelihood bootstrap analyses. The branching pattern for the deeper nodes of clade 1 was well supported in terms of bootstrap values. The topology was similar to that discussed in detail by Scholin et al. (1994, 1995) and here we included the sequence of a Vietnamese strain of *A. affine*. It formed an unresolved clade together with the three other strains included.

Except for the monophyly of *A. minutum* (≥80%), the topology of many of the terminal nodes in clade 2 were not well supported. The ANAKOHA strain was not part of the well-supported clade of *A. minutum* (Fig. 6). This was also indicated by the sequence divergence estimates (see above and Tables 3 and 4), indicating that re-investigation of this isolate is needed. The low sequence divergence among the *minutum* strains of this clade was clearly indicated by the very short branch lengths, and the topology shows more or less a polytomy.

4. Discussion

4.1. Morphology

Danish cells of *A. minutum* largely conform to Balech (1989) emended description except for the lack of a ventral pore. Together with the shape of the 1′ plate, it was the main distinctive feature separating *A. minutum* from the closely related *A. angustitabulatum*, originally described from New Zealand (Balech, 1995). The major right and left margins of the 1′ plate of *A. angustitabulatum* are almost straight and parallel, and the posterior right margin is shorter than in *A. minutum* (Balech, 1995). However, the present study showed considerable intra-clonal variation in the shape of the 1′ plate. Thus, several cells of the Danish material had a 1′ plate with parallel sides and a relatively short posterior right margin. Balech also noted that the length of the posterior right margin and the margin that borders the s.a. plate usually is of equal length, while the s.p. plate usually is longer than in *A. minutum*. These differences are also within the variation range observed in our material. We conclude that *A. minutum* and *A. angustitabulatum* are conspecific, although the toxin profile appears to differ between these two morphotypes in New Zealand waters (see below). A ventral pore was also absent in *A. minutum* strains from Brittany, e.g. strain AM89BM from Morlaix (Erard-Le Denn, 1997) and in the strain from Nantes (present study). Populations from southern England, Scotland and Ireland also lack a ventral pore, indicating that it may be a common feature in populations from these areas (see Table 5). However, a small fraction of the population from western Ireland had a ventral pore. Balech (1995) observed a ventral pore in material from Brittany, although the pore was very indistinct and only indented the 4′ plate (Balech,

Table 5
Presence or absence of a ventral pore, and toxin composition of different geographical isolates of *Alexandrium minutum*

Geographical location	Ventral pore	Toxic/(toxins)	Reference
Australia, Adelaide, Port River	+	+/GTX1–4	Hallegraeff et al. (1991)
Australia, Bunburry	?	+/GTX1–4	de Salas et al. (2001)
Australia, Newcastle	?	+/STX (neoSTX)	de Salas et al. (2001)
Australia, Portland	?	+	Hallegraeff (2002)
Australia, Shoalhaven	?	?	Hallegraeff (2002)
Croatia, Adriatic	?	?	Marasovic et al. (1995)
Denmark Korsør Nor	−	+/STX, GTX2 and 3, C1 and C2	This study
Egypt, Mediterranean	±	Fishkills	Halim (1960); Balech (1989); Ismael and Halim (2001)
England, Fal Estuary	−	+/ GTX2 and 3	Percy et al. (2002); personal observation
England, Fleet Lagoon	−	+/ STX, GTX2 and 3 (C-toxins)	Nascimento et al. (2002); personal observation
France, Brittany and Normandy	±	+/GTX2 and 3 (C-toxins)	Belin (1993); Ledoux et al. (1993); Balech (1995); this study
France, Toulon H., Mediterranean	?	+	Belin (1993)
India, Mangalore	+?	?	Godhe et al. (2001)
Ireland, Connemara coast	±	?	This study
Ireland, Cork Harbour	−	?	Gross, 1988; Orlova (personal communication)
Italy, Adriatic	+	+/ GTX2 and 3, STX?	Honsell (1993); Honsell et al. (1996)
Italy, Mediterranean, Gulf of Naples	+	?	Montresor et al. (1990)
Italy, Mediterranean, Sicily	+	?	Giacobbe and Maimone (1994)
Jamaica	+	?	Ranston (personal communication, personal observation)
Japan, Matoya Bay	+	?	Yuki (1994)
Kuwait, Kuwait Bay	+?	?	Glibert et al. (2002)
Malaysia, Tumpat	+	+/?	Usup et al. (2002a)
New Zealand, Bay of Plenty	+	+/neoSTX, STX, GTX1–4	Chang et al. (1997)
New Zealand, Marlborough Sounds	+	+/neoSTX, STX, GTX1–4	MacKenzie and Berkett (1997)
New Zealand, Whangarei Harbour	−	+/GTX1–4	Cembella and Taylor (1985); Taylor et al. (1995), as *A. angustitabulatum*
Norway, Oslofjorden	+	?	Balech and Tangen (1985), as *A. excavatum*
Portugal, Obidos Lagoon	+	+/GTX1–4	Balech (1995); Franco et al. (1995); this study
Scotland, Loch Roag, Lewis Island	−	?	Bresnan (personal communication)
Spain, Catalan, Mediterranean	+	+	Delgado et al. (1990); Balech (1995); Vila et al. (2001)
Spain, Galicia	+	+/GTX1–4	Franco et al. (1994, 1995)
Spain, Mallorca	+	+/GTX1–4 (C-toxins)	Forteza et al. (1998)
Sweden, Skagarak	±	?	Kuylenstierna and Karlson (2000) (http://www.marbot.gu.se/sss/ssshome.htm)
Taiwan, shrimp pond	+	+/GTX1–4	Su and Chiang (1991) as *A. tamarense*; Hwang and Lu (2000)
Thailand, Prakarn R. Estuary	+	+/GTX1–4	Piumsomboon et al. (2001)
Tunisia, Tunis Bay	+	?	Daly Yahia Kefi et al. (2001)
Turkey, Bay of Izmir	+	?	Koray and Buyukisik (1988); Balech (1995)
USA, New York	+	?	Balech (1995)
Vietnam, Don-Dien, shrimp pond	+	+/GTX1 and 4	Yoshida et al. (2000)
Vietnam, Hue and Nha Trang	+	?	Nguyen and Larsen (submitted)

1995, plate 1, Fig. 38). Balech and Tangen (1985), in their description of *Alexandrium excavatum* from Oslofjorden (here considered synonymous with *A. tamarense*), included a SEM micrograph (their Fig. 2) that most likely represents *A. minutum* with a distinct ventral pore. The occurrence of both *A. minutum* and *A. angustitabulatum* at the same localities in New Zealand, e.g. Whangarei Harbour and Bream Bay (Taylor et al., 1995; Hay et al., 2000) and in Swedish waters (Kuylenstierna and Karlson, 2000), also indicates that the ventral pore may not be a stable feature. In the original description of *A. minutum*, a ventral pore was not noted by Halim (1960), and Balech (1989) regarded it as being overlooked. However, considering the apparent variability of this character a ventral pore may have been absent in the material studied by Halim. Speculations on the presence of the pore are hampered by lack of knowledge regarding its function (if any) and of factors determining its presence. The LSU sequence data did not differentiate between populations with or without ventral pore.

An analogy to *A. minutum* occurs between *A. tamarense* and *A. fundyense*. The presence of a ventral pore is also used as the main distinctive character separating these two species, *A. tamarense* possessing a pore and *A. fundyense* lacking it (Balech, 1995). The two different morphotypes are interspersed geographically along the east coast of North America and Canada, though in some areas like the Gulf of Maine only *A. fundyense* occurs (Anderson et al., 1994). Mating experiments have shown that the two species are sexually compatible and produce viable offspring. However, the ventral pore was apparently not inherited in a Mendelian pattern as only one of 21 progeny cultures had a prominent pore. Dominance could be excluded, as dinoflagellates are haploid. It was suggested that the ventral pore was 'a quantitative character whose expression is affected by more than one gene' (Anderson et al., 1994). Still one would expect that a higher percentage of the progeny would display a ventral pore, and therefore other unknown factors most likely affect the expression of this character. Interestingly, Kim et al. (2002) observed cells with or without ventral pores in clonal cultures of *A.* cf. *catenella*.

The ventral pore has been considered a significant species-specific character (e.g. Balech, 1995). However, its apparent variability in *A. minutum* (this study), *A. tamarense/fundyense* (Anderson et al., 1994) and *A.* cf. *catenella* (Kim et al., 2002) makes this character questionable. The morphological species concept within *Alexandrium* is presently challenged. Thus, the molecular phylogenetic studies of Scholin and co-workers (e.g. Scholin et al., 1994) have shown that species of the *A. tamarense* species complex (*A. tamarense*, *A. fundyense* and *A. catenella*) group according to their geographic origin rather than to their morphotypes.

The Danish strains displayed considerable variation in the shape of the s.a. plate, from almost isodiametrical to significantly wider than length. The latter feature was used previously to distinguish *A. lusitanicum* from *A. minutum* (Balech, 1985, 1995). A similar variability of the s.a. plate has been previously noted in Galician strains of *A. minutum*, and in *A. lusitanicum* strain A18, the culture used by Balech for the original description of this species (Franco et al., 1995). In view of the apparent variability of this feature, we agree with Franco et al. (1995) that *A. lusitanicum* and *A. minutum* are conspecific. This is further supported by LSU sequence data.

4.2. Toxins

The toxin composition of the Danish strain of *A. minutum* was basically identical to the toxin profiles from other northern temperate populations, although STX was not detected in isolates from Brittany and the Fal Estuary (Table 5). This particular profile may be characteristic for this geographical region, but Honsell et al. (1996) found a similar toxin composition in populations from the Adriatic Sea. Otherwise, the most common or geographically widespread toxin composition of *A. minutum* seems to be GTX1–4 (Table 5). Isolates from New Zealand and eastern Australia differ by the presence of neoSTX, though only traces of this toxin are present in the latter isolates (MacKenzie and Berkett, 1997; de Salas et al., 2001). One New Zealand isolate differed significantly in its LSU sequence (see below). Interestingly, the isolate, originally referred to as *A. angustitabulatum* (=*A. minutum*, this study), did not produce neoSTX and STX but had a toxin composition identical to *A. lusitanicum* (=*A. minutum*, this study) (Cembella and Taylor, 1985; Taylor et al., 1995, see Table 5). The toxin profile may therefore be used to distinguish between these two morphotypes

in New Zealand waters. It is noteworthy that the *angustitabulatum* morphotype, i.e. cells lacking a ventral pore, possesses the typical *minutum* toxin composition, whereas the typical *minutum* morphotype, with a ventral pore, has an atypical toxin profile.

Earlier studies suggested that the relative toxin composition is a stable character, applicable for differentiating populations from different geographical regions (Cembella, 1998, and references therein). However, a recent study showed considerable inter-clonal variations in toxin composition of *A. tamarense* strains isolated from the same localities in Japan, and in strains established from the same cyst (Ichimi et al., 2002). This was suggested to be due to genetic recombination, the toxin profiles of natural populations merely reflecting the 'average' profile of all cells. Other studies have shown that considerable variations may occur within a single clone, depending on growth conditions (e.g. Boczar et al., 1988; Anderson et al., 1990). But, as pointed out by Cembella (1998), this variability may be more apparent than real and typically occurs in senescent cells or in stressed cultures, e.g. under nutrient limitation. This is also true for our isolate, which produced significantly more STX in the early stationary than in the late exponential growth phase. Nevertheless, the suite of toxins remained the same and this seems to be valid for all *Alexandrium* isolates investigated so far. In a recent study of a subclone of *A. minutum*, the A18 strain from Portugal originally referred to as *A. lusitanicum* (Anderson, personal communication), no detectable levels of toxins were found (Martins et al., 2002). This is to our knowledge the first example of loss of toxicity in an *Alexandrium* culture, though we have also observed that toxic *A. minutum* isolates become considerably less toxic after prolonged culturing (Franco, personal observation).

4.3. Molecular data

Partial LSU sequences did not distinguish between *A. minutum* morphotypes with and without a ventral pore and/or with differences in toxin profiles. Information on toxin profile and morphotype was unknown to us for many of the sequenced *A. minutum* strains retrieved from GenBank. However, strains A18 and Al1V both possess a ventral pore and we suspect that all the strains from the French coast of the English Channel lack a ventral pore like the Danish strain GHmin04. Erard-Le Denn (1997) noted that *A. minutum* (AM89BN) from Morlaix lacked a ventral pore, and we did not observe a ventral pore in the strain from Nantes. Strains from the English coast of the Channel also lack the ventral pore (see Fig. 7 and Table 5). The small divergence values among LSU sequences is perhaps not surprising as most of the analyzed strains originate from Europe, except one from Australia and one from New Zealand. The inclusion of the Australian strain among the European strains was somewhat surprising, and a European origin of this strain should be considered. In fact these strain, the Danish strain and the French AM89BM strain from Morlaix, were identical in the sequences examined. Perhaps a more rapidly evolving DNA sequence like ITS or AFLP markers may separate populations within a more restricted area, as well as strains with different morphology and toxin signatures. Interestingly, using LSU and ITS sequences, it has been shown that Australian *A. minutum* populations form two groups: a western Australian group with toxin profiles very similar to those of European populations (dominated by GTX1 and 4), and a 'Tasmania-New Zealand' group with the toxin profile dominated by STX and neoSTX (de Salas et al., 2001). Previous investigations based on LSU sequences grouped the New Zealand ANAKOHA isolate outside the group of European strains (Walsh et al., 1998; Guillou et al., 2002). In the study by Walsh et al. (1998), the ANAKOHA strain grouped with *A. ostenfeldii*, and showed a large sequence divergence compared to other *A minutum* strains. The ANAKOHA (AF033532) isolate represent either strain ANAKOHA A or ANAKOHA B (MacKenzie, personal communication), previously studied by MacKenzie and Berkett (1997). Based on their published micrographs, the isolates appear to represent the *A. minutum* morphotype.

Our molecular data support synonymy of *A. minutum* and *A. lusitanicum*, as also suggested by their morphology and toxin signature (see also Franco et al., 1995). Previous molecular studies grouped *A. lusitanicum* together with *A. minutum* (Scholin et al., 1994; Guillou et al., 2002). This is not surprising since all investigations have used the same isolate, though referred to under different strain designations. The strain was isolated from the Obidos Lagoon in Portugal more than 40 years ago (see Silva and Faust,

Fig. 7. Geographical distribution of *Alexandrium minutum*. Black circles (●) refer to morphotypes with a ventral pore, black squares (■) to morphotypes lacking a ventral pore, and open circles (○) to areas where both types have been observed (see Table 5).

1995), and used by Balech (1985) for describing *A. lusitanicum* (Franco et al., 1995).

Clustering of the *Alexandrium* species in two clades (Fig. 6) is evident, but morphological characters to define these clades seem to be lacking. The positioning of *A. pseudogonyaulax* outside clade 2 is perhaps not surprising considering its peculiar paratabulated resting cyst, which differs markedly from the smooth-walled cysts of other *Alexandrium* species (Montresor, 1995). The vegetative life cycle of *A. pseudogonyaulax* involves division cysts (Montresor, 1995), very similar to those observed in *F. subglobosum* (von Stosch, 1969, as *Helgolandinium subglobosum*; Skovgaard, 1996). Balech (1995) established two subgenera, *Alexandrium* and *Gessnerium*, distinguished by contact or separation between the first apical plate and the pore plate. Molecular data do not support this distinction, as *A. pseudogonyaulax*, *A. margalefii* and *A. insuetum*, all of the subgenus *Gessnerium* sensu Balech, do not group together (Fig. 6). Recently, M. Yoshida (Laboratory of Coastal Environmental Sciences, Nagasaki University, personal communication) used the shape of the posterior sulcal plates to identify *Alexandrium* species, and distinguished between four different types (type A–D). Usup et al. (2002b) found good agreement between these types and the phylogenetic tree based on LSU sequences. Our phylogenetic analyses also support these types (indicated in Fig. 6). Only *A. pseudogonyaulax* (not included in the analysis of Usup et al., 2002b) does not group with *A. margalefii*, although both belong to type B.

4.4. Biogeographical considerations

A. minutum is globally widespread but lacks in polar or subpolar regions (Fig. 7). Its absence from the African and South American continents may be due to lack of monitoring. However, it scarcity on the North American continent appears to be real, considering that extensive monitoring has carried on for several decades. The preferred habitat of *A. minutum* seems to be confined water bodies with low turbulence, high residence time and a high anthropogenic impact, for example harbours (e.g. Cannon, 1990; Giacobbe et al., 1996; Vila et al., 2001; present study). Such areas may

not receive the same attention as areas with extensive mariculture, and *A. minutum* may therefore escape normal monitoring except during massive blooms. This could be an explanation for its apparent absence in North America. Interestingly, Garcés et al. (2000) suggested that the increase of toxic events in the Mediterranean Sea may be due to increased exploitation of the coastlines for recreational purposes, leading to an increase in nutrient loads and to construction of artificial confined areas like harbours, breakwaters, etc.

The population structure and distribution pattern of *A. minutum* is not easy to understand. This may be explained by the relatively few strains of *A. minutum* examined (compare with the known distribution map in Fig. 7) and also by possible human-assisted transport of cysts in ships' ballast water. However, there is some indication of a European ribotype and a very different New Zealand ribotype, which perhaps includes western Australian populations. Population genetic studies of the *A. minutum* group by molecular markers are still in their infancy, future investigations may reveal the same geographical distribution pattern as in the *tamarense* complex.

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