

Morphological variation of two *Alexandrium* species responsible for paralytic shellfish poisoning in Southeast Asia

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Abstract

We examined in clonal cultures the morphological variation of two toxic dinoflagellates, *Alexandrium tamiyavanichii* and *A. minutum*, responsible for paralytic shellfish poisoning events in Southeast Asia. The Malaysian strain of *A. tamiyavanichii* had either a straight or oblique posterior margin on the first apical plate, and a triangular, square to trapezoid-shaped precingular part of the anterior sulcal plate. Morphological variability was also detected in the shape of the first apical plate, its connection to the apical pore, and the position of the ventral pore. In *A. minutum*, the Malaysian strains showed variation in the sixth precingular plate and the anterior sulcal plate. The length:width ratio of the sixth precingular plate varied from one to two. An anterior sulcal plate with a long left extension also occurred in both of the Malaysian strains. Several characters that have been extensively used in the identification of *Alexandrium* species have been proven quite variable.

Keywords: *Alexandrium minutum*; *Alexandrium tamiyavanichii*; morphology; paralytic shellfish poisoning; toxicity.

Introduction

The taxonomy of the genus *Alexandrium* Halim relies primarily on detailed descriptions of the thecal morphology of vegetative cells (Taylor 1984, Balech 1985, 1995). A consensus on the genus designation was reached in 1989 at the International Conference on Toxic Marine Phytoplankton held in Sweden (Balech 1990, Steidinger and Moestrup 1990). However, the species concept within the genus continues to be a subject of debate (Balech 1985, Taylor 1985, Steidinger 1990, Scholin 1998).

Alexandrium tamiyavanichii Balech and *A. minutum* Halim are the only two species of the genus *Alexandrium* reported so far to have caused paralytic shellfish poisoning (PSP) events in coastal regions of Southeast Asia

(e.g., Kodama et al. 1988, Usup et al. 2002a). In this region, *A. tamiyavanichii* was reported initially as *Protogonyaulax cohorticula* (Balech) Taylor (Kodama et al. 1988) and, subsequently, as *Alexandrium cohorticula* (Balech) Balech (Ogata et al. 1990). Balech re-examined specimens of *A. cohorticula* from the Gulf of Thailand and found some morphological differences that supported the designation of a distinct species, *A. tamiyavanichii* (Balech 1994, 1995). He also recorded the presence of this species in Manila Bay, Philippines, and the Andaman Sea of southwestern Thailand (Balech 1995). Morphological differences between *A. cohorticula* and *A. tamiyavanichii* were minor. The two species shared almost the same general thecal morphology, cell size and capability of forming chains of cells, whereas variations in the morphology of the anterior sulcal plate (s.a.), the posterior margin of the first apical plate (1') and the sulcal list projection have been used to distinguish the two species (Balech 1995).

Alexandrium minutum was recorded later in Southeast Asia and its importance was elevated by harmful outbreaks in Malaysia (Lim et al. 2004) and the Philippines (Bajarias et al. 2003). This species was described from Alexandria harbor, Egypt by Halim (1960), and a detailed description of the species was later provided by Balech (1989). *Alexandrium minutum* and its closely related taxa, *A. lusitanicum* Balech, *A. angustitabulatum* Taylor and *A. andersoni* Balech were distinguished by variation in several morphological characters (Balech 1995). *Alexandrium lusitanicum* was distinguished from *A. minutum* by the left margin extension of the anterior sulcal plate into the cingulum, while *A. angustitabulatum* was characterized by the lack of a ventral pore and *A. andersoni* by an irregular shape of the posterior sulcal plate (s.p.) (Balech 1995). A question remains as to whether or not these morphological characters warrant the separation of distinct species.

Some characters in *Alexandrium minutum* and its closely related species were proven to be unstable by Hansen et al. (2003). Recently, two new species, *A. camurascutulum* MacKenzie et Todd (MacKenzie and Todd 2002) and *A. tamutum* Montresor, Beran et John (Montresor et al. 2004), were described. These species also have a close relationship to *A. minutum*, but are distinguished by a different morphology of the sixth precingular plate (6''), which is wider in *A. camurascutulum* (MacKenzie and Todd 2002) and almost equal in width and length in *A. tamutum* (Montresor et al. 2004).

Our understanding of phenotypic variation within species of the genus *Alexandrium* is poor, and there are relatively few reports on intraspecific variability (Franco et al. 1995, Delgado et al. 1997, Hansen et al. 2003). It is difficult to assess phenotypic variability in field popula-



Figure 1 Map showing sampling locations of Sebatu and Tumpat in peninsular Malaysia.

tions (Morales et al. 2002), whereas the study of clonal cultures allows assessment of the range in the phenotypic diversity within cells that are genetically identical. In this study, the morphological variability of *A. tamiyavanichii* and *A. minutum* populations from Southeast Asia was examined using clonal strains.

Materials and methods

The Malaysian strain of *Alexandrium tamiyavanichii* used in this study was established from plankton samples collected from Sebatu in the southern part of the Strait of Malacca (Figure 1). The Malaysian strains of *A. minutum* were collected during a bloom in September 2001 at Tumpat on the northeastern coast of the Malaysian Peninsula (Lim et al. 2004). Samples were collected with a 20 μm mesh plankton net and brought back live to the laboratory. Clonal cultures were established by single cell isolation (Table 1). All cultures were grown in ES medium (Kokinos and Anderson 1995) and incubated at a light intensity of 140 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ provided by fluorescent tubes under 14:10 h light:dark cycle at a temperature of 25°C.

Cells were harvested by centrifugation at 3000 rpm for 5 min and preserved in 4% formalin solution. Prior to microscopical examination, cells were stained with 1% calcofluor white (Fritz and Triemer 1985) and examined

under an ultraviolet light on an Olympus BX51 microscope (Olympus, Melville, USA). Digital images were captured using a Pixera Penguin 600XL cooled CCD camera (Pixera Corporation, Los Gatos, USA). Images captured were further enhanced using the software ImageJ 1.30 (National Institutes of Health, USA, <http://rsb.info.nih.gov/ij>). Cell dimension was determined from a total of 50 randomly selected cells and mean values were calculated.

Results and discussion

The morphology of the thecal plates in the Malaysian strain of *Alexandrium tamiyavanichii* agreed with the description of Balech (1994). Cell diameter ranged from 32 to 49 μm , and chains made up of eight cells occurred in culture. The first apical plate (1') was rhomboidal with a direct connection to the apical pore complex (APC) (Figure 2A–C). The posterior margin of the 1' plate was slightly concave (Figure 2C,D). The ventral pore (vp) was present on the anterior right margin of the 1' plate. The apical pore (Po) was oval with a large anterior attachment pore located at the anterior right margin of the comma (Figure 2F). The absence of an anterior attachment pore was observed in some specimens from the Malaysian strain (Figure 2F). The s.a. plate was long and had a tri-

Table 1 Strains of *Alexandrium tamiyavanichii* and *Alexandrium minutum* used in this study.

Species and strains	Location	Isolation date	Collector	References for genetic characterization
<i>Alexandrium tamiyavanichii</i> AcMS01	Sebato, Malacca	October 1997	Po-Teen Lim	Usup et al. 2002b Leaw et al. 2005
<i>Alexandrium minutum</i> AmKB02 AmKB06	Tumpat, Kelantan	September 2001	Po-Teen Lim	Usup et al. 2002b Lim et al. 2004 Lim et al. in press

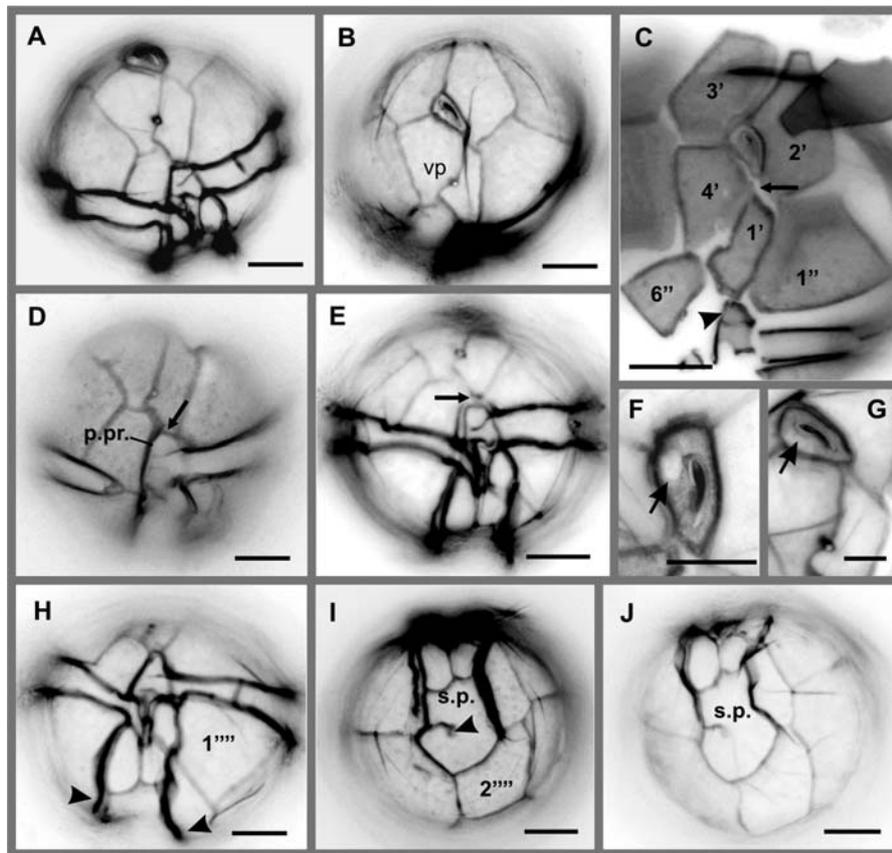


Figure 2 Light micrographs of *Alexandrium tamiyavanichii* from Southeast Asia.

(A) Ventral view of a typical cell. (B) Apical view of cell, vp: ventral pore. (C) Position of the apical pore complex with epithecal plates. Note the indirect connection of the first apical plate (1') with the apical pore (arrow) and the trapezoid shape of the precingular part (arrowhead). 2' 3' 4': second, third, fourth apical plates; 1'', 6'': first, sixth precingular plates. (D) An oblique posterior end of the 1' plate (arrow) and a triangular shape of the precingular part (p.pr.). (E) First apical plate (1') with a straight posterior margin (arrow). (F, G) Close-up of the apical pore (Po) with and without an anterior attachment pore (arrow). (G) Narrow posterior end of apical pore. (H) Sulcal plates and the first antapical plate (1''') with the sulcal list (arrowheads). Projection of the sulcal list to the posterior part of cell. (I) Long posterior sulcal plate (s.p.) with a posterior attachment pore (arrowhead) connected by an irregular furrow. 2''': second antapical plate. (J) Cell showing a relatively long s.p. plate. Scale bars: 10 μm (Figures A–E, H–J), 5 μm (Figures F and G).

angular (21%, $n=24$) to trapezoidal-shaped precingular part (p.pr.) (79%, $n=24$) (Figure 2A,C–E,H), which was also reported in specimens from the Gulf of Thailand (Fukuyo et al. 1988). In some specimens, the transverse rib did not extend to the right margin of the s.a. plate and formed an oblique fold (Figure 2A,E). The s.p. plate was long with a large posterior attachment pore connected by an irregular furrow to the right margin of the plate which was wider in its mid-portion (Figure 2I,J). Some specimens lacked the posterior attachment pore (Figure 2J). The second antapical plate (2''') was wide (Figure 2I). All these features have been used to distinguish *A. tamiyavanichii* from other *Alexandrium* species (Balech 1995). There was, however, some intraclonal variation in the specimens, including features that are characteristic of other species.

In some specimens, the 1' plate had a straight posterior margin (Figure 2E), while in others a longer and square precingular part (p.pr.) of the sulcal plate had a straight transverse rib (Figure 2C). The shape of the precingular part (p.pr.) of the s.a. plate and the posterior margin of the 1' plate is amongst the characters distinguishing *Alexandrium cohorticula* from *A. tamiyavanichii* as well as the shape of the Po (Balech 1995). The *cohor-*

ticula-type Po is longer and more sharp-pointed, and the comma is positioned slightly ventrally, while the *tamiyavanichii*-type Po is wider and the comma is located centrally. These features were present in the same clonal culture in this study (Figure 2C,F,G). The size of the sulcal list was variable in all cultures observed. Both less developed (65%, $n=23$) and strongly developed sulcal lists (35%, $n=23$) were found in specimens from the same culture.

Specimens with the 1' plate disconnected from the Po occurred in the Malaysian strain (14%, $n=22$). The 1' plate was slightly disconnected from the Po by a fine suture (Figure 2C). In some specimens, the 1' plate was narrow, pentagonal and completely disconnected from the apical pore. This feature was not reported in either *Alexandrium cohorticula* or *A. tamiyavanichii* (Balech 1995). Interestingly, a closely related species in Balech's description of *Alexandrium* species with a Po directly and indirectly connected to the 1' plate was identified as *A. kutnerae* (Balech) Balech (Balech 1995). This latter species shares several characteristic features with *A. cohorticula* and *A. tamiyavanichii*, such as the s.a. plate with a precingular part (p.pr.) and a rather developed sulcal list. The distinctive features differentiating *A. kutnerae* are the

large size of the cells, the disconnected 1' plate and the position of the ventral pore (Balech 1995).

The ribosomal genes (18S rRNA, 5.8S rRNA, domain 1–2 of 28S rRNA) and the internal transcribed regions (ITS1 and ITS2) sequence analyses of the Malaysian *Alexandrium tamiyavanichii* AcMS01 are well studied (Usup et al. 2002b, Leaw et al. 2005). Unfortunately, cultures of *A. cohorticula* and *A. kutnerae* are not available for molecular comparisons. Recently, several strains of *A. tamiyavanichii* from different localities were studied (Nagai et al. 2005, Ruiz Sebastian et al. 2005). The Japanese *A. tamiyavanichii* strains possessed 28S rRNA gene sequences identical to those of the Malaysian strain, and branched out as a monophyletic group with *A. tamarensis* CU13 and *A. fraterculus* forming the sister clade (Leaw et al. 2005) considered as species closely related morphologically to *A. tamiyavanichii/cohorticula* (Balech 1995). However, it is interesting to note that the South African strain has some degree of heterogeneity compared to the Malaysian and Japanese strains (Ruiz Sebastian et al. 2005).

Our results showed that the distinguishing morphological features used to differentiate between *Alexandrium tamiyavanichii* and *A. cohorticula* are phenotypic variants and do not support the current taxonomic position of *A. tamiyavanichii* and *A. cohorticula*. Therefore, we consider *A. cohorticula* as a valid synonym of *A. tamiyavanichii*.

The *Alexandrium tamiyavanichii* strain AcMS01 contains toxins GTX1-5, C1, C2, neoSTX, and STX, while GTX4+1 is the predominant congener (80% mol), followed by GTX3+2 (8%), GTX5 (6.4%), C1+2 (4.7%) and STX (1.6%) (Lim and Ogata 2005, Lim et al. 2006). Trace amounts of dcSTX and neoSTX also occur (<0.1%). The concentration of cellular toxin differs over the life cycle and with environmental conditions over a range from 80 to 160 fmol toxin cell⁻¹ (Lim et al. 2006). The toxin composition of the Malaysian strain is more similar to that of the Thai strains (Kodama et al. 1988) and some of the Japanese strains (Ogata et al. 1990) (Table 2). However, the western Japanese strain of *A. tamiyavanichii* has C1 and C2 as the predominant toxins, with cellular concentrations varying from 40 to 424 fmol cell⁻¹ (Nagai et al. 2005) (Table 2). *Alexandrium tamiyavanichii* was also reported from South Africa, with a close phylogenetic relationship to the Malaysian and Japanese strains, but with a rather low toxicity (<1 fmol cell⁻¹) (Ruiz Sebastian et al. 2005) (Table 2).

Our strains of *Alexandrium minutum* showed features typical of *A. minutum* (Balech 1989, 1995), with small, rounded cells of a diameter varying from 18 and 30 µm. The rhomboidal 1' plate had a ventral pore (vp) on the right anterior margin (Figure 3A,B) in almost all specimens studied (Table 3). In some specimens the 1' plate was apparently disconnected from the apical pore (Po). In some cells, the junction was hidden by the suture of two adjacent apical plates (Figure 3C), while in others the 1' plate was connected to the Po by a narrow extension of the anterior margin of the 1' plate (Figure 3D). The s.p. plate was wider than long, with a longer left posterior lateral margin. The anterior margin formed a "w" shape (Figure 3E–G). Both longer and wider right posterior sulcal plate (s.d.p.) occurred in specimens from the same culture (Figure 3E–G,I).

Table 2 Toxicity of *Alexandrium tamiyavanichii* from different geographical regions.

Strains (locality)	Toxins (% mol)										References	
	GTX4 + 1	GTX3+2	GTX5	GTX6	C1+2	C3	C4	neoSTX	dcSTX	STX		Toxin content (fmol cell ⁻¹)
AcMS01 (Malaysia)	+++ (79)	+	++ (6.5)	-	+	-	-	-	-	+	60–180	Lim et al. 2006
Chula 5 (Gulf of Thai)	+++ (85.1)	++ (9.9)	+	-	+	-	-	-	-	+	16200 ¹	Kodama et al. 1988
Chula 6 (Gulf of Thai)	+++ (77.8)	++ (11.2)	+	-	+	-	-	-	-	+	7500 ¹	Kodama et al. 1988
Chula 8 (Gulf of Thai)	+++ (74.3)	++ (2.5)	++ (7.7)	-	+	++ (8.0)	-	-	-	++ (7.3)	18.9	Ogata et al. 1990
MMBS8811-1 (Sagami Bay, Japan)	+++ (39.4)	++ (7.5)	++ (9.1)	-	++ (9.7)	+	++ (8.8)	-	-	++ (23)	3.7	Ogata et al. 1990
MMBS8811-3 (Sagami Bay, Japan)	+++ (48.3)	-	++ (13.3)	-	+	-	-	-	-	++ (23)	66.3	Ogata et al. 1990
CTCC23 (South Africa)	+	+	+++ (30)	-	++ (20)	-	-	+++ (30)	-	-	0.26 ²	Ruiz Sebastian et al. 2005
Western Japan	++	++	++	-	40	+	-	+	+	+	40–424	Nagai et al. 2005

+++ : >30% mol; ++ : 5–30%; + : <5%. ¹: number of cells per mouse unit, cells MU⁻¹; ²: STX eq., equivalent of Saxitoxin.

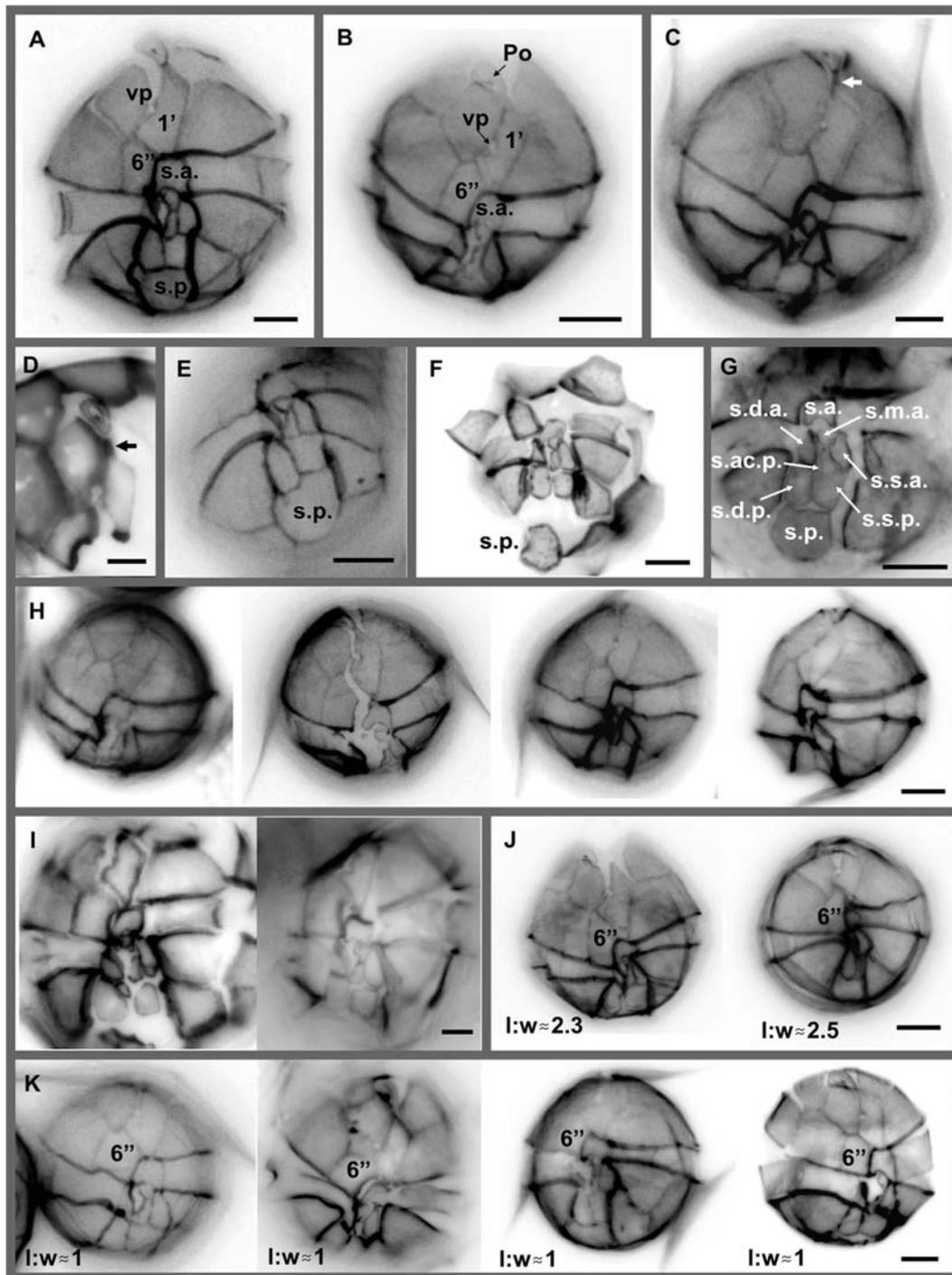


Figure 3 Light micrographs of *Alexandrium minutum* from Southeast Asia.

(A, B) Ventral views of typical cells. vp: ventral pore (arrow); Po: apical pore (arrow); 1': first apical plate; 6'': sixth precingular plate; s.a.: anterior sulcal plate; s.p.: posterior sulcal plate. (C) Cell with intercalary bands, note the disconnection of the 1' plate and the apical pore (arrow). (D) First apical plate connected to the apical pore by a narrow extension (arrow). (E, F) Typical *minutum*-type. s.p.: posterior sulcal plate. (G) Detail of the sulcal plates. s.s.a.: left anterior lateral plate; s.s.p.: left posterior lateral plate; s.m.p.: posterior median plate; s.m.a.: anterior median plate; s.d.p.: right posterior lateral plate; s.d.a.: right anterior lateral plate; s.ac.a.: anterior accessory plate; s.ac.p.: posterior accessory plate. (H, I) Cells with different widths of the anterior sulcal plate. (J) Cells with reduced sutures between the fourth (4'') and the sixth (6'') precingular plate. Cells also show remarkably long 6'' plates. l:w: length to width ratio of the 6'' plate. (K) Cells with a wide 6'' plate. l:w: length to width ratio of the 6'' plate. Scale bars: 5 μ m.

A high degree of variation was observed in the shape of the s.a. plate and sixth precingular plate (6'') (Table 3). Cells with both wide and narrow s.a. plates were found in cultures (Figure 3H). The width of the s.a. plate is a characteristic feature separating *Alexandrium lusitanicum*

from *A. minutum* (Balech 1995). Our results, however, showed that the shape of the s.a. plate is variable, and agree with the findings of Franco et al. (1995), who proposed that *A. lusitanicum* and *A. minutum* were conspecific. In our culture specimens, the length:width ratio of

the 6" plate was >1.5 (Figure 3A,B,H,J). Interestingly, some specimens with wider and larger 6" plates were also observed in our culture material (Figure 3K). Recently, *Alexandrium tamutum* was described based mainly on the shape of the 6" plate (Montresor et al. 2004). Our results showed that the shape of the 6" plate was variable in clonal cultures of all strains examined (Table 3). Cells with a wider 6" plate might represent a different morphotype of *A. minutum*. A wider 6" plate was observed to be variable from narrow (thin) to wide in different strains of *A. minutum* (Lilly et al. 2005). A combined molecular and morphological analysis also showed that this character might be homoplastic (Leaw et al. 2005). Both Malaysian strains of *A. minutum*, AmKB02 and AmKB06, were closely related to the other Asian Pacific strains, but differed from the European and Australian strains (Leaw et al. 2005, Lim et al. in press).

All strains of *Alexandrium minutum* used in the present study produce PSP toxins. Both Malaysian strains contained GTX4 and GTX1 as the dominant toxin congeners, with more than 90% mol, and other toxin congeners GTX2, GTX3 and STX, present in trace amounts (Lim and Ogata 2005). A similar toxin composition was also observed in the Thai strains, with an absence of STXs (Piumsomboon et al. 2001). Previous studies have shown that the dominance of GTX1 and GTX4 is a characteristic of the *A. minutum* toxin profile (Hallegraeff et al. 1991, Franco et al. 1994, Chen and Chou 2002). However, a marked difference in toxin profiles of *A. minutum* has been reported (Hansen et al. 2003). Recently, non-toxic strains of *A. minutum* were also reported from various locations (Tillmann and John 2002, Martins et al. 2004). By constructing a dendrogram using the toxin profiles of *A. minutum* from different geographical origins, the strains clustered into two major groups (Figure 4). The first group consisted of strains from Malaysia, Thailand, Australia, Vietnam, Spain, Portugal, Taiwan and New Zealand, while the second group comprised strains from France and Denmark. The toxin composition of this latter group is characterized by a high proportion of C1 toxin (44–60%). Geographical divergence of toxin profiles has also been reported in many species of *Alexandrium* (Cembella et al. 1987, Anderson et al. 1994, Cembella

Table 3 Morphological variation in *Alexandrium minutum* strains (as percentage of cells).

Strains	AmKB02	AmKB06
Cell dimension (μm)	20–30	20–30
Sixth precingular plate (6")		
Wide (length:width=1)	30%	22%
Narrow (length:width >1)	70% (n=36)	78% (n=36)
Anterior sulcal plate (s.a.)		
Wide	39%	37%
Narrow	61% (n=40)	63% (n=47)
Ventral pore (vp)		
Present	90%	95%
Absent	10% (n=48)	5% (n=30)

and Destombe 1996, Yoshida et al. 2001) and *Gymnodinium catenatum* Graham (Oshima et al. 1993).

While certain morphological characters remained stable throughout this study, several characters that have been used to distinguish species were variable. Recently, an approach combining morphological and molecular data has shown that morphological characters that were previously regarded as taxonomically important are homoplastic (Leaw et al. 2005). Several molecular studies have been carried out on *Alexandrium* species to infer the biogeographical origins (Scholin and Anderson 1994, Scholin et al. 1994, Hansen et al. 2003, Ruiz Sebastian et al. 2005) and the phylogenetic positions of the species (Usup et al. 2002b, Leaw et al. 2005, Lilly et al. 2005). However, the application of molecular phylogenetic approaches cannot provide strong evidence for the delineation of species. Morphological variation in *A. tamiyavanichii* and *A. minutum* has important taxonomic implication for the current description of *Alexandrium* morphospecies. However, we still lack sufficient information to review the taxonomy of these species, due to the absence of culture specimens from type localities. This indicates the need for a reconsideration of morphological variability in a more comprehensive manner for species designation, particularly in the genus *Alexandrium*. Observations of specimens from type localities will be crucially important.

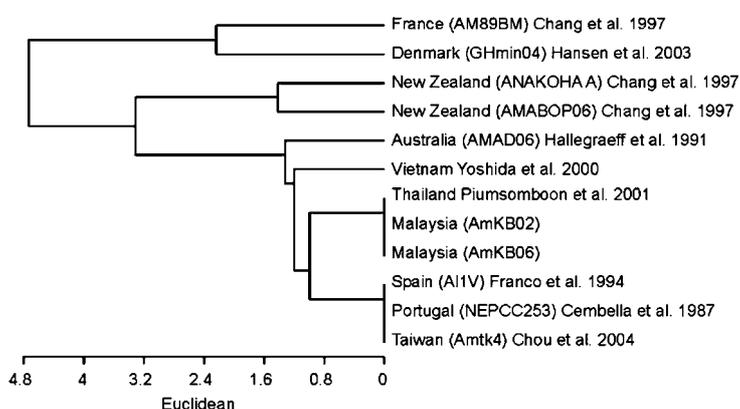


Figure 4 Dendrogram based on toxin profiles of *Alexandrium minutum* strains from different geographical regions. Euclidean distance was used to determine distances. The average linkage method of unweighted pair-groups method average (UPGMA) was used to construct the dendrogram.

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