

First Record of Marine Dinoflagellate, *Alexandrium tamutum* (Dinophyceae) from Malaysia

Kon, Nyuk Fong¹, Hii, Kieng Soon¹, Tan, Toh Hii¹, Yek, Leh Hie¹, Lim, Po Teen², Leaw, Chui Pin^{1*}

¹ Institute of Biodiversity and Environmental Conservation (IBEC), University Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan, Sarawak, Malaysia.

² Faculty of Resource Science and Technology (FRST), University Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan, Sarawak, Malaysia.

*cpleaw@ibec.unimas.my, chuipinleaw@gmail.com (corresponding author)

ABSTRACT Several species of dinoflagellates in the genus *Alexandrium* are known to be toxic, and have been associated with paralytic shellfish poisoning (PSP) in Malaysia. These *Alexandrium* species showed high morphological similarity among the toxic and non-toxic species, and detailed observation of the thecal plate's arrangement is required for precise species identification. Co-occurrence of the toxic and non-toxic species has complicated the plankton monitoring of PSP. In this study, a clone of *Alexandrium* species was established from plankton samples collected from Kota Belud, Sabah. The specimen was observed under epi-fluorescence microscope, and nucleotide sequences of the nuclear-encoded ribosomal RNA gene obtained. Morphologically, the clone showed relatively wide and large sixth precingular plate (6'') compared to that of *A. minutum*. The posterior sulcal plate (Sp) is similar to that of *A. minutum*, which is wider than long. The first apical plate (1') is irregularly rhomboidal with a small ventral pore (vp) present on its right margin. The morphological characters resembled to the species description of *A. tamutum*. Phylogenetic analysis of the ITS rDNA region also revealed a monophyly of this clone with other strains of *A. tamutum*, and separating them from the *A. minutum* clade. Species-specific sequence signatures of *A. tamutum* were obtained *in silico*, which could be as potential oligonucleotide probe regions for species detection by using molecular tool. This represents the first report of *A. tamutum* found in Malaysian waters.

(Keywords: *Alexandrium tamutum*, *A. minutum*, Sabah, Malaysia, thecal plates)

INTRODUCTION

Harmful algal bloom (HAB) is a natural phenomenon due to the increase in density of phytoplankton resulted in adverse effect to the ecosystem. Contamination of biotoxins from the phytoplankton may lead to shellfish poisoning when the toxins are transferred to human via the shellfish vectors. In Malaysia, the most frequently reported seafood intoxication associated with algal toxins is paralytic shellfish poisoning (PSP) [1]. PSP is caused by the consumption of contaminated shellfish such as mussels, clams, oyster, scallop or other filter feeders [2, 3]. The PSP toxin, collectively called Saxitoxins (STXs), is a group of toxin family that blocks the voltage gated sodium channels in mammalian nerve cells and inhibits nerve conduction, which may lead to paralysis of the neuromuscular system [4].

Bloom of the marine dinoflagellate, *Pyrodinium bahamense* var. *compressum*, in Brunei Bay and

the subsequent PSP incidence in Sabah [5], was the first record of HABs and PSP in Malaysia. Whilst in Peninsular Malaysia, poisoning case was first reported in 1991 when people consumed the green mussel, *Perna viridis*, cultured at a mussel farm in Sebatu, Malacca. In September 2001, poisoning case including one fatality was reported from Tumpat, Kelantan, a coastal lagoon of the east coast of Peninsular Malaysia. The intoxication was due to the consumption of contaminated benthic bivalve, *Polymesoda* sp. (local name, 'lokan') [6].

In this study, a marine dinoflagellate from the genus *Alexandrium* was morphologically and molecularly characterized. Samples were obtained from Kota Belud, Sabah, Malaysia, and clonal cultures of the dinoflagellate species were established. Both preserved and field samples were observed under an epi-fluorescence microscope. Clonal cultures were further analyzed based on the nucleotide sequences of ribosomal RNA genes (rDNA). Genomic DNA was extracted, and the

rDNA was amplified prior to sequencing. The sequences obtained were analyzed and used for phylogenetic reconstruction. Species-specific sequence signatures of *A. tamutum* were also obtained *in silico*.

MATERIALS AND METHODS

Algal cultures

Plankton samples were collected from Kota Belud, Sabah, using a 20 µm-mesh plankton net. Sampling was undertaken during high tide, with salinity of ~30 psu. Field *in situ* parameter i.e. temperature, salinity and pH of the seawater was collected. Live samples were kept in 1 L bottles in cool condition and brought back to the laboratory for single-cell isolation. A portion of samples (1/3) were preserved in acidic Lugol's solution.

Samples were sieved through a 20 µm-mesh sieve and back-washed to a petri dish for single-cell isolation by micropipetting technique under an IX51 inverted light microscope (Olympus, Melville, USA). Clonal cultures were established in a 96-well microplate containing filtered sterile seawater. Cultures established were grown in ES-DK medium [7], and maintained at 25±0.5 °C under a 12:12 h light:dark photoperiod in a SHEL LAB temperature-light controlled growth chamber (SHEL LAB, USA).

Species identification

Morphological observation was performed with an epifluorescence microscopy. Identification of the species was based on cell shape and theca plate tabulation. Fixed samples were stained with 1% Calcofluor white solution (Fluka, Japan), and the nuclei were stained with SYTOX Green DNA stain (Invitrogen, Life Technologies, USA). Stained samples were observed under an Olympus IX51 epifluorescence microscope (Olympus, Melville, USA) with UV filter sets. Images were captured with a cooled CCD camera (SIS Colorview F12, Germany).

DNA extraction, rDNA amplification and sequencing

Mid-exponential growth phase cultures were harvested by centrifugation at 2,800 rpm for 10 min. The cell pellet was then suspended in distilled water, followed by cetyltrimethylammonium bromide (CTAB) extraction as described in [8, 9]. Cells were lysed by adding equal volume of 2× CTAB lysis buffer containing 50 mM CTAB, 14 mM NaCl, 10 mM Tris-base, 20 mM EDTA,

and 1% mercaptoethanol. The mixture was extracted with chloroform: isoamyl alcohol (C:I, 24:1), and subsequently phenol:chloroform:isoamyl alcohol (P:C:I, 25:24:1). Two volumes of absolute ethanol and 1/10 volume of 3M sodium acetate were added to precipitate the DNA, followed by centrifugation at 13000 rpm for 10 min. DNA pellet was rinsed with 70% cold ethanol and then dissolved in 30 µL TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0). Genomic DNA samples were kept overnight at 4°C to totally dissolve the DNA before stored at -20°C for further analysis.

The internal transcribed spacer region (ITS1-5.8S-ITS2) of rDNA was amplified by polymerase chain reaction (PCR) using the primer pair, ITS1F (5' TCGTAACAAGGTTTCCGTAGGTG-3') and ITS1R (5' ATATGCTTAAGTTCAGCGGG-3') [8]. Gene amplification was performed as described in [8, 9]. In brief, the amplification condition is as follow: initial denaturation at 95°C for 5 min, denaturation at 94°C for 18 seconds, annealing at 55°C for 18 seconds, followed by elongation at 72°C for 1 min and final elongation at 72°C for 7 min. PCR products were subjected to further purification prior to DNA sequencing by the sequencing service laboratory (1st Base, Selangor, Malaysia), using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequencing was performed in both strands.

Sequence analysis and taxon sampling

Sequences were evaluated by using the Basic Local Alignment Search Tool (BLAST) program [10], and analyzed by ABI Sequence Scanner ver. 1.0 (ABI BioSystem, USA). Ambiguous bases were determined by comparing both forward and reverse strands of each strain. The reverse strands were reversed complemented by using BioEdit version 6.0.7 [11] and pairwise-aligned with the forward strands using Clustal-X [12]. These were followed by assignment according to the IUPAC nucleotide code.

Taxon sampling was achieved by blasting in the nucleotide database, GenBank, in conjunction with the National Center of Biotechnology Institute (NCBI) to acquire related sequences. The selected sequences were saved as FASTA format for further analysis.

Phylogenetic analyses

Phylogenetic analyses was performed by using Phylogenetic Analysis Using Parsimony (PAUP) ver.

4.0b10 [13] with the maximum likelihood (ML) and maximum parsimony (MP) algorithms used to estimate the phylogeny.

For ML analysis, Modeltest ver. 3.06 [14, 15] with α value of 0.01 was used to identify the hierarchical likelihood ratio so that the best model of evolution can be determined. The evolutionary model was selected for the sequences data set and the ML parameters were used to reconstruct the ML tree. ML was performed by heuristic search with tree bisection-reconnection (TBR) branch swapping and 10 random additions.

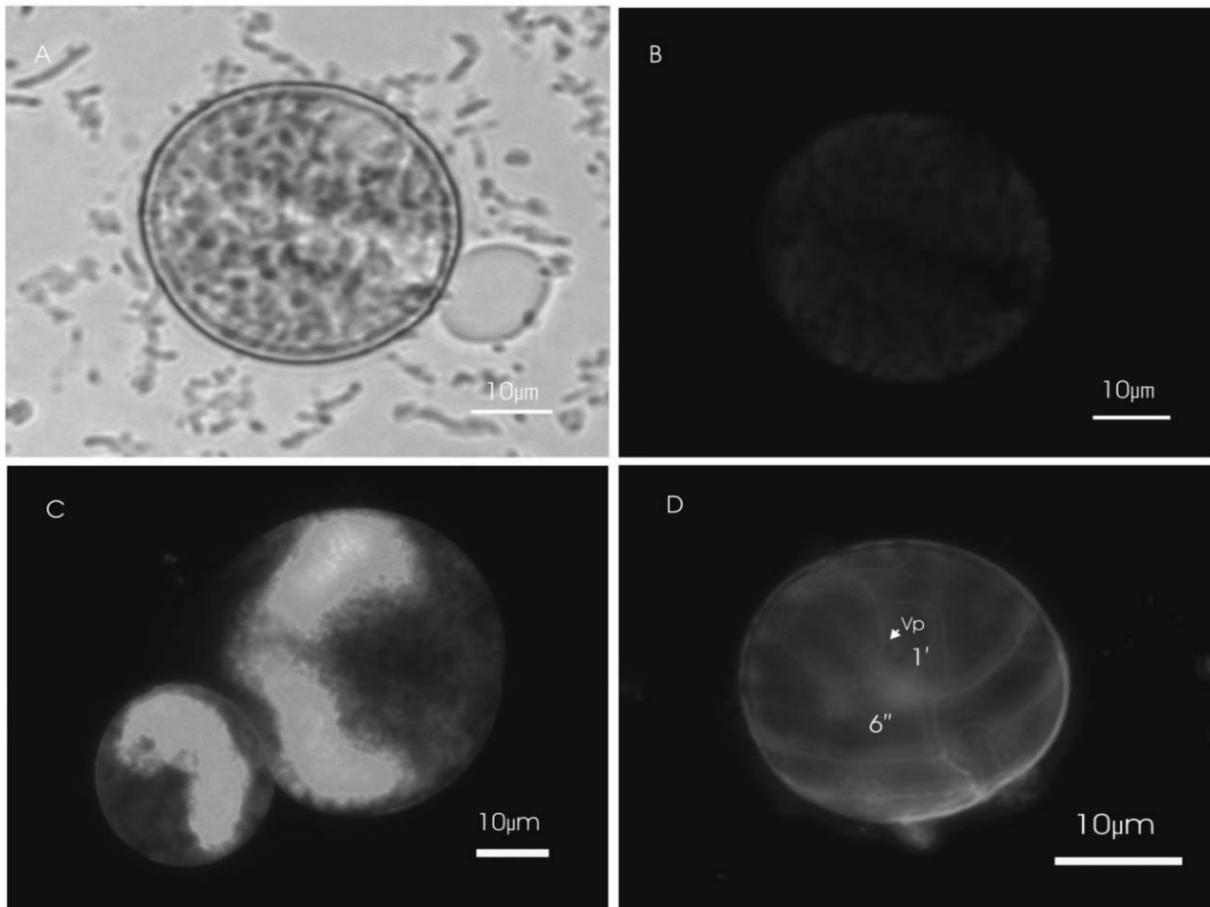
MP was performed by heuristic search of 1,000 random addition and TBR branch swapping. Bootstrapping [16] was performed on both ML and MP analyses with 1,000 replication ($P < 0.001$) to determine the confidence limits of tree topology using TBR branch swapping algorithm.

Sequence signatures assignment

The ITS sequence of *A. tamutum* was aligned and compared to sequences of all other *Alexandrium* species retrieved from GenBank to search for the signature regions. Species-specific sequence signatures were identified by using ARB package.

RESULTS AND DISCUSSION

Among the species of *Alexandrium*, *A. tamutum* showed high morphological similarity to its closely related toxic species, *A. minutum*. Microscopic cell observation without pre-staining often is difficult to discriminate the two species. Only detailed thecal plate arrangement and the shapes revealed upon thecal staining could be used to differentiate the two species. The morphological characteristic of the sixth precingular plate (6'') is one



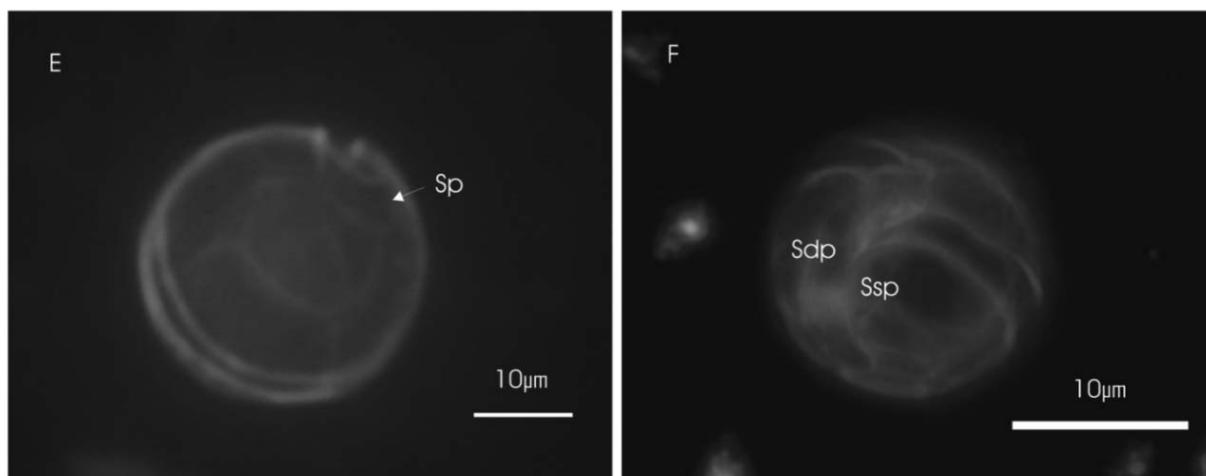


Figure 1. *Alexandrium tamutum* strain AuKA01 from Kota Belud, Sabah. (A) Bright field. (B) Cell showing chlorophyll pigments. (C) SYTOX green-stained cells showing the arc shaped nuclei. (D) Cell with thin thecal plates showing rhomboidal 1' and wide 6''. Note the ventral pore (vp). (E) Antapical view of cell with wider than long posterior sulcal plate (Sp). (F) Cell showing the sulcus with deep and excavated cingulum.

of the main diagnostic features used to delineate the two species [17].

Morphological characterization of *A. tamutum*

Cells are round and small, with 9–25 μm long and 9–33 μm wide (**Figure 1**). Chlorophyll pigments present indicating the cells are photosynthetic (Figure 1B). The fairly large nucleus with chromosomes that are permanently condensed can be readily seen after SYTOX Green staining (Figure 1C). The thecal plates are thin and smooth. Cingulum is deep and excavated, with its left end shifted anteriorly (Figure 1D). The first apical plate (1') is irregularly rhomboidal, with a small ventral pore (vp) present on its right margin (Figure 1D). The sixth precingular plate (6'') is wider than long, and adjacent to 1' (Figure 1D). The posterior sulcal plate (Sp) on the hypotheca is wider than long (Figure 1E). The epi-fluorescence image clearly showed the sulcal area of *A. tamutum*, with both right posterior sulcal plate (Sdp) and left posterior sulcal plate (Ssp) (Figure 1F).

Alexandrium tamutum resembled *A. minutum* in its relatively small cell size, rounded or elliptical [17]. However, large *A. tamutum* cells have a slightly pentagonal shape which is similar to the outline of small-sized *A. tamarensis* [17]. *Alexandrium andersoni* is another small-sized species which is difficult to distinguish it from *A. tamutum* and *A. minutum*. Both *A. andersoni* and *A. minutum* are PSP toxin producers [18], but *A. tamutum* was reported as non-toxic [17].

Several *Alexandrium* species share similar cell outline and size ranges. Thus, it is difficult to distinguish *Alexandrium* species solely based on cell size and shape. Identification at species level requires detailed investigation of the pattern of their thecal plates. *Alexandrium tamutum* differs from *A. minutum* by its relatively wide and large 6'', whereas *A. minutum* has narrower and smaller 6'' [17]. The large 6'' of *A. tamutum* is similar to that of *A. tamarensis* [17].

Shape of the sulcal posterior plate (Sp) has been suggested as a diagnostic character to delineate species of *Alexandrium* [19]. The Sp of *A. tamutum* is similar to that of *A. minutum* which is wider than long, while the Sp is longer than wide in *A. tamarensis*. Both *A. tamutum* and *A. minutum* have rhomboidal 1' plate. However, the vp of that in *A. tamutum* is located in the median/upper part of the margin [17], whilst in *A. minutum* it is located on the posterior end of the anterior right margin of plate 1' [20].

ITS rDNA phylogeny of *Alexandrium*

The ITS region of rDNA of *A. tamutum* was successfully amplified for the strain AuKA01. Blast search revealed an e-value of 0.0 with a total of six hits to *A. tamutum* in the nucleotide database of GenBank (NCBI). Closely related species of *A. tamutum* were included in the taxon sampling; they are *A. affine*, *A. andersoni*, *A. catenella*, *A. minutum*, *A. ostenfeldii*, *A. peruvianum*, *A. tamarensis* and *A. tamiyavanichii* (**Table 1**). *Pyrodinium bahamense* var. *compressum* was used as the outgroup.

Table 1. Sequences of ITS region retrieved from NCBI nucleotide database with species, strain and accession.

Species	Strain	Location
<i>Alexandrium affine</i>	AC-1	China
	AS-1	China
<i>A. andersoni</i>	CCMP 1718	Massachusetts, USA
<i>A. catenella</i>	PE01	Southern Chile
	A4	Daya Bay, China
	AD1	East China Sea, China
<i>A. minutum</i>	CBA64	Catalan Sea, Spain
	AMITA	Trieste, Italy
	Palmira1	Catalan Sea, Spain
	CBA28	Tyrrhenian Sea, Italy
<i>A. ostenfeldii</i>	AOFUN0905	Hokkaido, Japan
	AOFUN0904	Hokkaido, Japan
	ASBH01	China
<i>A. peruvianum</i>	IEO-VGOAMD12	Catalan Sea, Spain
	IEO-VGOAM10	Catalan Sea, Spain
<i>A. tamarensis</i>	04-197-30	Scotland, United Kingdom
	S06-010-01	Scotland, United Kingdom
<i>A. tamiyavanichii</i>	A1PSA	Porto Seguro, Brazil
	B2PSA	Porto Seguro, Brazil
	PSII	Porto Seguro, Brazil
<i>A. tamutum</i>	IEO-VGO616	Catalan Sea, Spain
	IEO-VGO617	Catalan Sea, Spain
	IEO-VGO615	Catalan Sea, Spain
	AL2T	-
	LBM-A5	North West Adriatic Sea, Italy
	IEO-VGO662	Tyrrhenian Sea, Italy

The resulting BI tree showed that AuKA01 strain obtained in this study was clustered together with other *A. tamutum* from GenBank (AM236857, AM236858, AM236859, AM236860, AM238452 and EU707497) (**Figure 2**). This further supported the species identification of strain AuKA01 as *A. tamutum*. The clade formed a sister group with *A. minutum* group (FR668134, FR668136, FR668140, FR668142). The molecular phylogenetic inference supported the morphological observation that *A. tamutum* was a sister taxon to *A. minutum*.

The MP analysis revealed 333 parsimony-informative characters, resulted in one parsimonious tree with a tree

length of 1,039 evolutionary steps, with the parsimony indices of consistency index (CI) = 0.7036, homoplasy index (HI) = 0.2964, retention index (RI) = 0.8229, and rescaled consistency index (RC) = 0.5790.

Bootstrap supports of the monophyly of *A. tamutum* were relatively high with MP, ML bootstraps of 81% and 78% respectively, and BI posterior probability (PP) of 1.0. The *A. minutum* clade was also supported by 100% for MP, 96% for ML and 1.0 for BI.

Although *A. tamutum* is closely related morphologically to *A. minutum*, the results of phylogenetic inferences

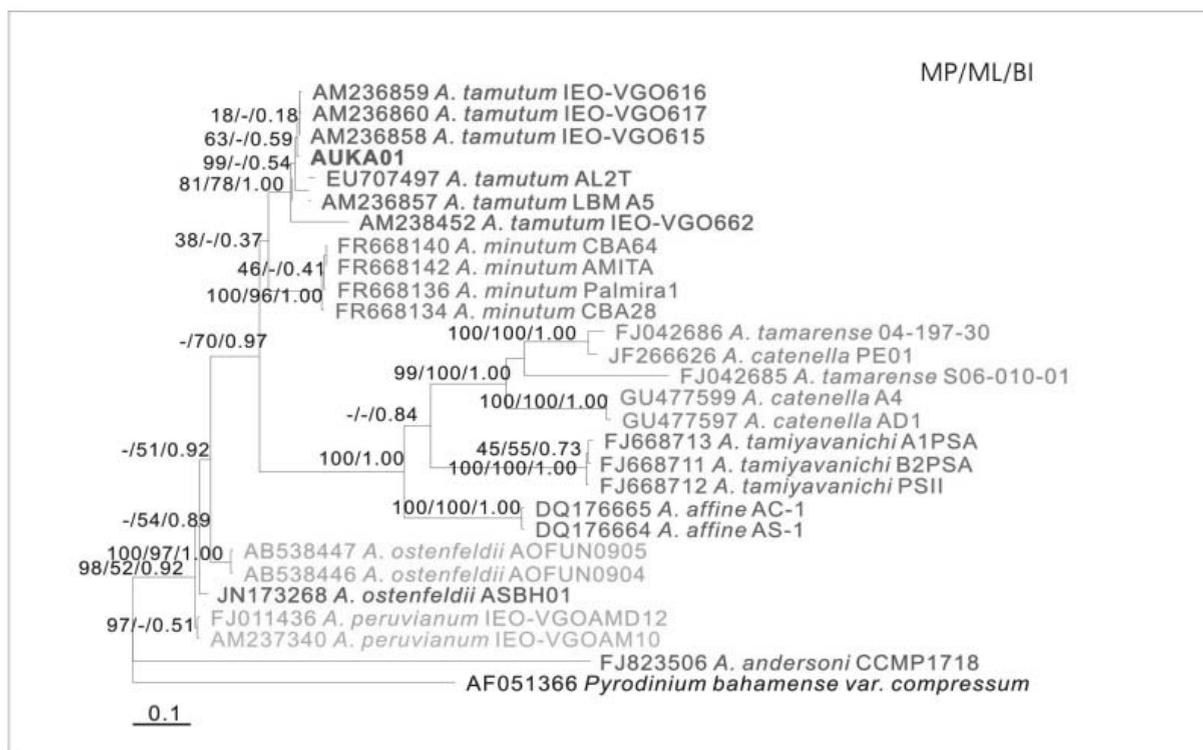


Figure 2. Phylogenetic tree of *Alexandrium* species derived from Bayesian analysis (BI) of ITS sequences. *Alexandrium tamutum* from Kota Belud, Sabah used in this study are in boldface. Bootstrap supports of MP/ML/BI are shown at the internal nodes. Scale bar = 0.1 substitution per site.

strongly supported the split of *A. tamutum* as a single clade, while *A. minutum* formed another clade (Figure 2).

Sequence signature

The ITS sequence of *A. tamutum* was aligned with ITS sequences of other *Alexandrium* species retrieved from the GenBank to search for the signature regions.

By using ARB program, several regions of sequences with nucleotides length of 25bp and 100% hits were identified. These regions were identified as species-specific to *A. tamutum* based on the nucleotide polymorphism to the non-target species. **Table 2** shows the E-value and number of hits of the nblast search result. Three signature regions were proposed as the DNA signature of *A. tamutum* (**Figure 3**).

Table 2. nBlast search of signature regions revealed the respective e-value and the number of hits

Signature	Uppermost Blastn hit against NCBI nr-database	e-value	Number of hits
AGGGUGGCAUGGCUUGCAAUAGCAA	<i>A. tamutum</i>	2e-04	6
AUGCUGCUGCAUUGGACACACGCGC	<i>A. tamutum</i>	2e-04	5
AUUUGCUCGAGGGUGGCAUGGCUUG	<i>A. tamutum</i>	2e-04	5

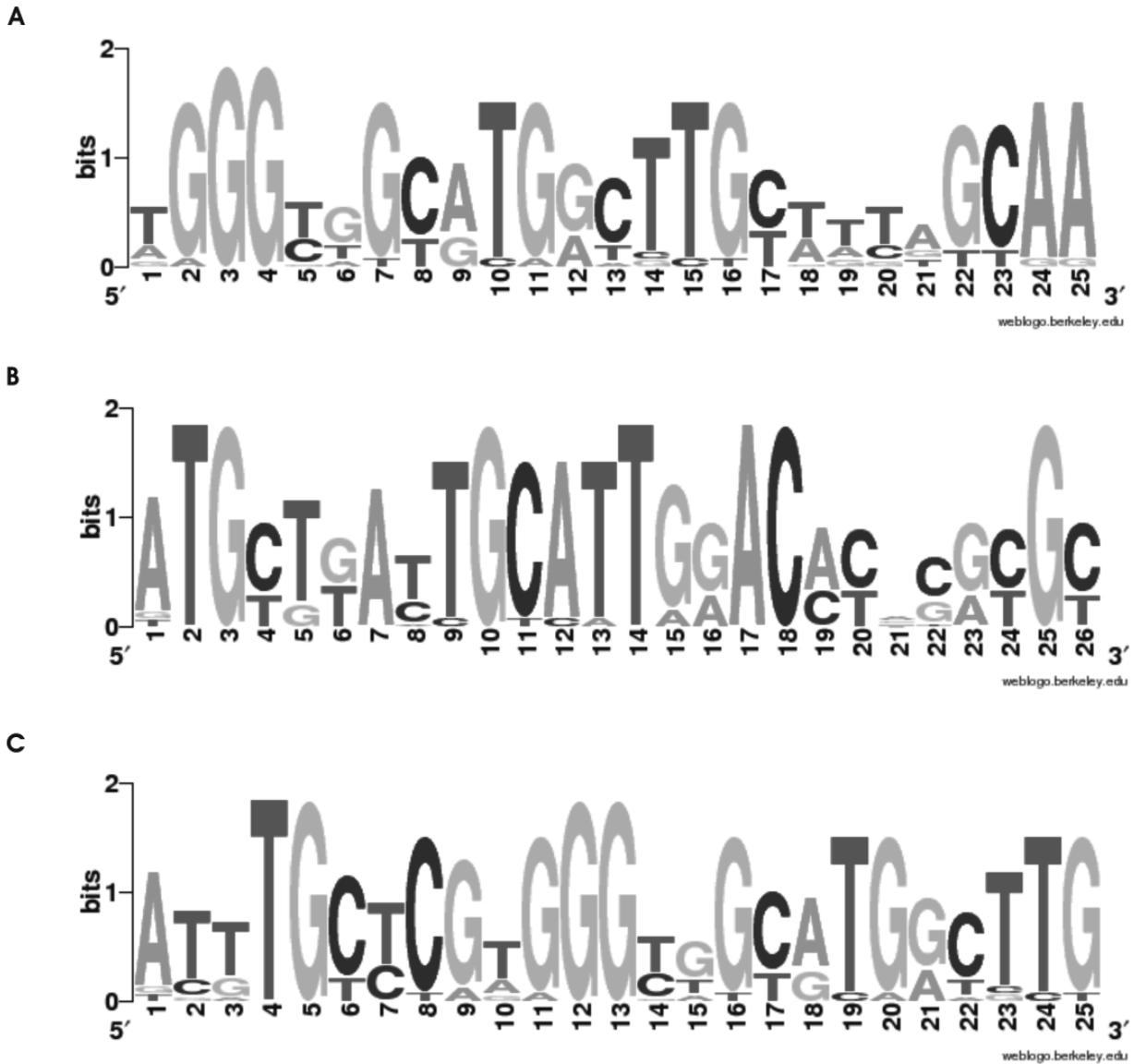


Figure 3. Three possible sequence logos of signature region of *A. tamutum* predicted in this study

CONCLUSION

Culture of *Alexandrium* from Kota Belud, Sabah was identified and designated as *Alexandrium tamutum* based on the rhomboidal first apical plate (1'), wide sixth precingular plate (6'') and wide posterior sulcal plate (Sp). The phylogenetic analyses also supported the species identity based on the evidence of monophyly of taxa identified as *A. tamutum*. This represents the first report of *A. tamutum* found in the Malaysian waters. Species-specific sequence signatures of *A. tamutum* were obtained *in silico*. The species-specific sequence

signatures of *A. tamutum* obtained in this study can be used to develop molecular probe for rapid detection of the species.

REFERENCES

1. Lim P. T., Leaw C. P., Usup G. and Ahmad A. (2002). Probable toxin producer responsible for the first occurrence of paralytic shellfish poisoning on the east coast of peninsula Malaysia. *Malaysian Applied Biology* **31**(2): 29-35.

2. Shumway S. (1990). A review of the effect of algal blooms on shellfish and aquaculture. *Journal of World Aquaculture Society* **21**: 65-104.
3. Shumway S. E., Sherman-Caswell S. and Hurst J. W. (1988). Paralytic shellfish poisoning in Maine: Monitoring a monster. *Journal of Shellfish Research* **7**: 643-652.
4. Doucette G. J., Logan M. L., Ramsdell J. S. and Dolah F. M. V. (1997). Development and preliminary validation of a microtiter plate-based receptor binding assay for paralytic shellfish poisoning toxins. *Toxicon* **35**: 625-636.
5. Roy R. N. (1976). Red tide and outbreak of paralytic shellfish poisoning in Sabah. *Medical Journal of Malaysia* **31**(3): 247-251.
6. Lim P. T., Leaw C. P. and Usup, G. (2004). First incidence of paralytic shellfish poisoning on the east coast of Peninsular Malaysia. In Phang, S. M., Chong, V. C., Ho, S. S., Mokhtar, N., Ooi, J. L. S. (eds.) *Marine Science into New Millennium: New perspectives and challenges*. University of Malaya Maritime Research Centre, Kuala Lumpur, pp. 661-667
7. Kokinos J. P. and Anderson D. M. (1995). Morphological development of resting cysts in cultures of the marine dinoflagellate *Linguladinium polyedrum* (= *L. machaerophorum*). *Palynology* **19**: 143-166.
8. Leaw C. P., Lim P. T., Ahmad A. and Usup G. (2001). Genetic diversity of *Ostreopsis ovata* (Dinophyceae) from Malaysia. *Marine Biotechnology* **3**(3): 246-255.
9. Leaw C. P., Lim P. T., Ng B. K., Cheah M. Y., Ahmad A. and Usup G. (2005). Phylogenetic analysis of *Alexandrium* species and *Pyrodinium bahamense* (Dinophyceae) based on theca morphology and nuclear ribosomal gene sequence. *Phycologia* **44**: 550-565.
10. Altschul S. F., Gish W., Miller W., Myer E. W. and Lipman D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.
11. Hall T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.
12. Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. and Higgins D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876-4882.
13. Swofford D. L. (2000). *PAUP*: phylogenetic analysis using parsimony (* and other methods)*. Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, USA.
14. Posada D. and Crandall K. A. (1998). Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.
15. Posada D. and Crandall K. A. (2001). Selecting the best-fit model of nucleotide substitution. *Systematic Biology* **50**: 580-601.
16. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.
17. Montresor M., John U., Beran A. and Medlin L. (2004). *Alexandrium tamutum* sp. nov. (Dinophyceae): A new, non-toxic species in the genus *Alexandrium*. *Journal of Phycology* **40** (2): 398-411.
18. Ciminiello P., Fattorusso E., Fiorino M. and Montresor, M. (2000). Saxitoxin and neosaxitoxin as toxic principles of *Alexandrium andersoni* (Dinophyceae) from the Gulf of Naples, Italy. *Toxicon* **38**: 1871-1877.
19. Usup G., Leaw C. P., Ahmad A. and Lim P. T. (2002). Phylogenetic relationship of *Alexandrium tamayavanichii* (Dinophyceae) to other *Alexandrium* species based on ribosomal RNA gene sequences. *Harmful Algae* **1**: 59-68.
20. Balech E. (1995). *The Genus Alexandrium halim (Dinoflagellata)*. Sherkin Island Marine Station, Cork, Ireland.
21. Schneider T. D and Stephens R. M. (1990). Sequence logos: A new way to display consensus sequences. *Nucleic Acids Research* **18**(20): 6097-6100.