

# Rapid Detection of Toxic Dinoflagellate, *Alexandrium minutum* (Dinophyceae) Using Fluorescence *In Situ* Hybridization (FISH)

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**ABSTRACT** Harmful algal blooms (HABs) are phenomena known as sudden increase in Microalgal population that cause seafood poisoning in human and impact to marine ecosystem. Dinoflagellates from the genus *Alexandrium* had been known to be responsible for paralytic shellfish poisoning (PSP) toxins with the production of sodium blocking neurotoxins collectively called saxitoxin (STX). Species identification in this genus is based on thecal plate tabulation and minute morphological character, which is often hard to observe during regular plankton monitoring that require taxonomic expertise. Hence, a molecular detection approach using whole-cell fluorescence *in situ* hybridization (FISH) was investigated in this study for the rapid detection of *Alexandrium*. Ribosomal DNA probes targeting the toxic *A. minutum* were designed *in silico*. Specificity and accessibility of designed probes were further verified *in silico* by comparing parameters that influenced hybridization kinetics. Species-specific probe which was designated as L-S-Amin-569-A-18 was synthesized and optimized using the clonal cultures of *A. minutum*. The results showed that the DNA probe had high specificity towards *A. minutum*, with no cross-reactivity towards other *Alexandrium* species. The whole-cell FISH coupled with the species-specific probe showed its potency as a rapid detection tool of *A. minutum* in Malaysian waters, which could be adopted in the national harmful algal monitoring program.

**(Keywords:** Paralytic shellfish poisoning (PSP); *Alexandrium minutum*, “fluorescence *in situ* hybridization” (FISH); DNA probe)

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## INTRODUCTION

Paralytic shellfish poisoning (PSP) is a type of seafood poisoning due to consumption of shellfish molluscs contaminated by neurotoxins, saxitoxin (STX). The toxins blocked sodium channels in mammalian nerve cells and thus prevent signal propagation along the neuron [1, 2]. Monitoring program on plankton and shellfish toxicity has been implemented in most countries affected by PSP as proactive measures in seafood safety. Early detection and enumeration of harmful algal species is pivotal in preventing human intoxication and impacts on ecological and economic aspects [2].

Worldwide distribution of toxic species in the genus *Alexandrium* has increased the severity of PSP. Co-occurrence of toxic *Alexandrium* species with non-toxic but morphologically highly similar further complicated the effort in plankton monitoring. In Malaysian waters, *A. minutum* and *A. tamiyavanichii* are responsible for PSP events in the Peninsular Malaysia [3, 4, 5, 6]. These

species have been found as part of the phytoplankton assemblages with non-toxic *A. leei* and *A. affine* [3, 7] and low toxic *A. tamarensis* [8].

Saxitoxins are tasteless, odorless, and toxicity remained stable after cooking processes. Therefore, a variety of detection techniques using molecular approaches such as fluorescence *in situ* hybridization (FISH) [9, 10], DNA microarrays, real-time quantitative PCR [11], and sandwich hybridization assays (SHAs) [12] have been employed for the rapid detection of harmful algal species [13].

Monitoring of toxic algae using morphological identification and conventional microscopic procedures is time consuming, and requires taxonomic expertise. *Alexandrium minutum* is difficult to differentiate among species as it was only characterized by minute morphological details in the thecal plates. Hence, FISH with chemiluminescent detection is a more suitable tool for rapid and reliable detection of harmful algae [10,

14, 15]. Moreover, this method requires a considerable shorter time compared to other methods.

In this study, species-specific oligonucleotide probe was designed *in silico* in large subunit ribosomal RNA using the ARB package. The designed probes were subsequently evaluated based on factors that influenced hybridization kinetics and efficiency. The optimum probe was then tested and applied on cultured *Alexandrium* species. Targeted cell were detected with the assistance of light and epi-fluorescence microscope equipped with a charge coupled device (CCD) camera. Probe optimization such as probe stability, probe intensity and cross reactivity of the probe was undertaken in order to validate the efficiency of probe.

## MATERIALS AND METHODS

### Algal cultures

Clonal cultures of *Alexandrium minutum* and several other *Alexandrium* species were grown in ES-DK medium [16] at  $25 \pm 0.5^\circ\text{C}$  under condition of 12:12h light : dark cycle, with photon flux of  $140\mu\text{mol photon m}^{-2}\text{s}^{-1}$ . Filtered natural seawater diluted to 15psu was used as medium base for *A. minutum* cultures, while for other *Alexandrium* species salinity of 30 psu was used. The medium pH was adjusted to 7.8-7.9 by adding 10% HCl.

### Morphological observation

All clonal cultures of *Alexandrium* species used in this study were confirmed based on morphological features such as shape of apical pore plate, presence of ventral pore on 1' apical plate and also the position of posterior and attachment pores as previously described [5, 6, 7, 17, 18]. Cells were fixed with 4% paraformaldehyde for not more than an hour, subsequently stained with 1% calcofluor white (Sigma) solution for 10 min, and finally viewed under an Olympus IX51 research microscope (Olympus, Tokyo, Japan) equipped with UV filter sets. Stained cells were analyzed and images were captured with the CCD camera.

### Phylogenetic reconstruction

Nucleotide sequences of the large subunit (LSU) ribosomal RNA gene of the species used in this study were retrieved from Genbank nucleotide database (NCBI). The LSU rDNA were initially aligned using Clustal X[19], and the alignment were further edited

manually using BioEdit ver 7.0[20]. Phylogenetic analyses were performed using PAUP\* ver. 4.0b10 [21].

### *In silico* rRNA-targeted oligonucleotide probes design

The rRNA-targeted oligonucleotide probes were designed *in silico* using PROBE Design Tool (PDT) of the ARB software package [22]. PDT searched the entire possible target sites and also probe sequences that are diagnostic for the selected species. Some parameters like hybridization efficiency assisted in selecting the most suitable probe sequence. The selected probe was compared against the whole database by using PROBE Match tool (PMT) of ARB software package. For confirmation, the unambiguous sequences were then blasted in BLAST search (Genbank, NCBI) [23] to check against all currently available sequences.

### Nomenclature of probe

In this study, the selected probe was chosen for probe synthesis for use in whole-cell FISH. The nomenclature of probe was adopted from [24]. The selected probe was designated as L-S-Amin-569-A-18.

### Whole-cell fluorescence *in situ* hybridization (FISH) optimization

Before performing the whole-cell FISH protocol, cells were counted using a Sedgewick-Rafter slide under a light microscope as to determine the suitable volume of cell suspension required for harvesting as well as microscopic detection. One milliliter subsamples were taken for triplicate counts.

Cells in the exponential phase were harvested by centrifugation ( $2000 \times g$ , 5 min) and fixed with 4% paraformaldehyde at  $4^\circ\text{C}$  for 1h. Cell pellets were dehydrated with an ethanol series (70% for 5 min, 90% for 5 min) at room temperature. Cells were spun down and pellets were rinsed with 1 mL of  $10\times$  PBS for 5 min (1–2 times) to remove excess ethanol, and followed by 1 mL  $25\times$  hybridization buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M TrisHCl, pH 7.8). The samples were then spun down again and supernatant discarded.

Cell pellet was resuspended in 200  $\mu\text{L}$  pre-warmed hybridization buffer [ $5\times$  SET, 0.1% (v/v) Nonidet P-40] containing 0.5 ng  $\mu\text{L}^{-1}$  of probes (species-specific probe, Universal eukaryotic probe, uniC and negative control probe, uniR). Mixtures were incubated for 45 min at  $55^\circ\text{C}$ . Subsequently hybridized samples

were centrifuged and supernatant with excess probe discarded. Cell pellets were rinsed with 1 mL pre-warmed 5× SET for 5 min to stop the hybridization reaction. Samples were spun again and cell pellet were resuspended in 200 µL 5× SET.

Total of 10 µL of each sample was transferred onto a microscope slide. Approximately 20 µL of Slow Fade Light (Invitrogen, CA, USA) was added to each sample. The slide was covered with a cover slip and observed under an Olympus IX51 epi-fluorescence inverted microscope (Olympus, Tokyo, Japan) equipped with UV filter sets. Fluorescent images were captured through CCD camera for image analysis.

## RESULTS

### Morphological observation of cultured *Alexandrium* species

All clonal cultures of *Alexandrium* species used in this study were confirmed based on the thecal morphology; they are *A. minutum* (AmKB06), *A. tamiyavanichii* (AcSm01), *A. affine* (AaSm01), and *A. tamutum* (AuKA01). Thecal plate tabulation of *Alexandrium* is Po, 4', 6'', 5''', 2''''', 6c and 9–10s according to Kofoidian plate formula [17]. Epi-fluorescence micrographs of *A. minutum* showed the narrow and long sixth precingular plate (6''), and a typical *minutum*-type anterior sulcal plate (s.a.) which is slight wavy in appearance (Figure 1A, B).

### LSU rDNA phylogeny of *Alexandrium*

The multiple alignments of 29 nucleotide sequences of LSU rDNA yielded 552 characters. Phylogenetic analyses (Maximum Parsimony, MP; Maximum Likelihood, ML; Bayesian Inference, BI) yielded identical tree topologies, with ML tree shown here (Figure 2). The topologies resolved two monophyletic clades, namely Clade 1 and Clade 2, respectively. *Alexandrium minutum* is closely related to *A. lusitanicum*, *A. insuetum*, *A. tamutum*, and *A. ostenfeldii*, forming a monophyletic group as Clade 1. On the other hands, *A. tamerense*, *A. catenella*, *A. affine*, *A. tamiyavanichii* and *A. fraterculus* formed another clade which is far more distant than *A. minutum*.

### Phylogeography of *A. minutum*

The *A. minutum* group were further analysed phylogenetically based on the geographical origin.

In this analysis, taxon sampling was restricted to *A. minutum* from different geographical origins. Total of 30 sequences were retrieved from the NCBI nucleotide database, with an alignment yielding 571 characters (including gaps). Two monophyletic clades of *A. minutum* were resolved in all the phylogenetic analyses (MP, ML, and BI) (Figure 3). *Alexandrium minutum* from Malaysia that used in this study has close relationship with *A. minutum* from China, Taiwan, New Zealand and USA. However, *A. minutum* from Italy, France, Spain, England or Ireland were split out as Clade 2.

### *in silico* rRNA oligonucleotide evaluation and probe design

For *in silico* probe design, the target species *A. minutum* species were retrieved from SILVA database. A total of 334 *Alexandrium* sequences were used. A total of 51 potential probe regions were selected *in silico* for *A. minutum* (Table 1). Four out of 51 potential probes showed 100% hybridization efficiency (HE), with Gibb's free energy values ( $\Delta G_{\text{overall}}^{\circ}$ ) less than the threshold -13 kcal/mol.

Parameters that influenced hybridization kinetics and stability of probe such as length of probe, melting temperature ( $T_m$ ), G+C contents are shown in Table 2. Three proposed probes (probe 2, 3, 4) are with lengths of 18-mer,  $T_m$  of 60°C and G+C contents of 66.7%. Probe 1 had slightly lower G+C content (61.1%) and lower  $T_m$  compared to others. Confirmatory test was performed with Blastn via NCBI nucleotide database to detect the potential matching target sequences in species from non-target and non-algal groups in the database. The results of the *in silico* search revealed an E-value of 1.0 which is considered as good probe specificity.

In this study, the selected probe region revealed high numbers of mismatches compared to other species of *Alexandrium* (Figure 4). The results showed probe 3 had the highest number of mismatches. Thus, the probe is selected for further probe synthesis and the probe was designated as L-S-Amin-569-A-18 according to Probe Nomenclature, with the sequence of 5' AGUCCCUUCCCCGUUGGC 3'.

### Mismatch analysis of *A. minutum* species-specific probe

The separation of two geographical groups of *A. minutum* based on our phylogenetic analyses in Figure 3 and others (e.g. [6, 25]) revealed a high genetic divergence

in *A. minutum*. In this study, a species-specific probe was designed to detect the populations that encountered in our waters. Thus, potential probe region targeting species in Malaysia and Taiwan (Clade 1) was chosen.

Mismatch analysis of the probe against all available *Alexandrium* sequences in the public databases (SILVA and GenBank) showed that no mismatch was found between the probe and the target *A. minutum* in the Pacific region (Clade 1). On the other hand, comparison of the probe regions against *A. minutum* of non-Pacific region (Clade 2) recovered as many as seven mismatches (Table 3).

### Stringency analysis

*in silico* analysis of formamide concentration showed no hybridization could occur between the probe and *A. minutum* from France, *A. minutum* in Clade 2 (non-Pacific region). The results further indicated that the probe selected has high hybridization efficiency (100%) and high specificity, thus this probe could be applied towards FISH without addition of formamide.

### Whole-cell fluorescence *in situ* hybridization (FISH)

Whole-cell FISH with FITC-labeled rDNA probe was performed with clonal cultures of *A. minutum*, with *A. tamiyavanichii* (AcSm01) and *A. tamutum* (AuKA01) as the non-target species. The universal eukaryotic probe, Uni C was acted as a positive control probe and when hybridized with the probe, *A. minutum*, *A. tamiyavanichii* and *A. tamutum* cells all showed clear green fluorescent signal (Figure 5A). As a negative control, the probe UniR was applied to the experiment and the results showed no green fluorescent signal from *A. minutum*, *A. tamiyavanichii* and *A. tamutum* cells (Figure 5B). However, some weak yellowish signal was observed within the cells which may be due to cell autofluorescence. On the other hand, the *A. minutum* species-specific probe [L-S-Amin-569-A-18] gave positive green fluorescent signal when hybridized with *A. minutum* (Figure 5C); the cell showed only green fluorescent at a specific region instead of whole-cell.

To confirm the specificity of the probe, cross-reactivity of the probe towards non-target species, *A. tamiyavanichii* and *A. tamutum* were tested. When the probe applied on *A. tamiyavanichii* and *A. tamutum* cells, the cells did not show any green fluorescent (Figure 5D, 5E). The results clearly indicated that the probe is species-specific and thus confirmed the specificity of the probe designed.

### Optimization of FISH

To optimize the condition of FISH, several parameters were optimized in this study to obtain the maximum efficiency of probe binding. These included hybridization temperature, time of fixation and chemical compounds used in hybridization, for instances formamide concentration, washing buffer concentration, probe concentration, fixative.

Good fixation of cells is pivotal in the initial procedure of whole-cell FISH. In this study, two common fixatives used in FISH were tested for their efficiency. Our results showed that paraformaldehyde (4%) fixed the dinoflagellate cells better compared to the modified saline ethanol (data not shown). Optimum probe concentration was determined by varying the final concentration in hybridization. In this study, the concentration at 200 ng  $\mu\text{L}^{-1}$  was shown to have higher intensity compared to other diluted concentrations of probe. Optimum hybridization temperature used in this study ranged from 45°C to 58°C. Cells hybridized at temperature of 55°C were selected due to higher probe specificity and intensity.

## DISCUSSION

The LSU rDNA sequences of *Alexandrium* spp. indicated that the D1-D2 region could provide a species-specific genetic marker for the genus *Alexandrium*. Classification based on phylogenetic relationships within this genus based on the sequences retrieved from D1-D2 region rDNA is congruent with morphological taxonomy [6, 18, 19, 20, 25], and thus could be used to assist in the probe design [26, 27]. DNA probe sequence selected must be specific to differentiate the target organisms not only from closely related species but also from a great number of other non-related organisms in the field [28].

In the present study, several common technical problems were observed in applying whole-cell FISH. We present here some solutions for the problems occurred. Under epi-fluorescence microscopy, small yellowish pyrenoid-like spots in the cells often appeared after UV excitation, and sometimes could be misleading as positive results of hybridization. This could be distinguished from the positive green fluorescence signal where the whole cells are often fluorescently stained. The identity of this fluorescent organelle is still unclear though [29]. The occurrence of auto-fluorescence in *Alexandrium* has caused masking of hybridization signals with FISH assays. However, auto-fluorescence of *Alexandrium* spp. could be greatly reduced by ethanol series after fixation.

Paraformaldehyde has been widely used for fixation owing its easier cell wall permeabilization for binding of target molecules in cytosol and DNA probe molecules[28]. Of various fixation agents tested such as paraformaldehyde, glutaraldehyde and modified saline ethanol, paraformaldehyde was found to be the most suitable fixative for FISH (with a fixation time of not more than 1h). Glutaraldehyde (final concentration of 0.1%) caused higher fluorescence background and also lower signal strength of cells compared to paraformaldehyde. Modified saline ethanol has been reported as effective at permeabilizing cell wall of microalgal species include diatom (especially *Pseudo-nitzschia* species) and also thecate or non-thecate dinoflagellates [30]. This solution allows strong cell permeabilization and allows probe to access target molecules[31]. However, the fixative did not work well with *Alexandrium* spp. as strong auto-fluorescence was observed in this study.

To obtain the highest probe reactivity (fluorescence intensity) of labelled cells without background noises, optimum hybridization temperature and conditions were determined in this study. Hybridization depends on the ability of oligonucleotide to anneal to a complementary mRNA strand just below its melting point ( $T_m$ ) where half of the oligonucleotide presented in single-stranded condition. Application of FISH protocol using higher temperature has been considered as effective way to decrease the non-specific binding. This also increases the accessibility of targeted rRNA to oligonucleotide DNA probe.

In this study, FISH protocol was optimized at 55-58°C, which was considered high as compared with the usual hybridization temperature (45°C or even lower). High temperatures lead to conformation of secondary and tertiary structure of rRNA as well as to ensure both oligonucleotide probe and targeted fragments of rRNAs strands allocate at correct orientation. Hybridization temperature was closely related to fluorescence intensity because temperature not only affected dissociation of probe but also affects conformation of targeted rRNA. Hybridization temperature that was significantly below the theoretical  $T_m$  easily binds to non-targeted nucleic acid.

DNA targeted probe can be applied either as a whole-cell FISH where the signal was found throughout the cytoplasm due to ribosomal distributions or the probe is merely hybridized to nuclear-encoded DNA that the signal were only observed from nuclei[32].

Applying DNA probe may be advantageous for quantifying cells compared to RNA probe. Probe hybridized with rRNA and rDNA differ in their perspectives. rRNA are highly abundance in cells but rRNA abundance varies among cells physiological condition and thus show varying intensity, leading to errors when quantification of target cells. Although rDNA is less abundance, the advantages of using rDNA is that the number of targeted cells will not vary depends on physiological or environmental conditions (except during cell divisions) and hence it can be used for quantification of target cells.

DNA probes have great potential as diagnostic tools because they are capable to distinguish strain-specific genotypes because genetic signatures should not be affected when cells experience changing environmental conditions[33]. DNA-targeted probe is hybridized to nucleus of the cells, showing that DNA probe has more specificity as compared to RNA probe. DNA probe had advantage compared to RNA probe in term of storage. DNA can be stored longer in low temperature compared to RNA probe which needs to be frozen in -20°C since RNA is easier to degrade.

## CONCLUSION

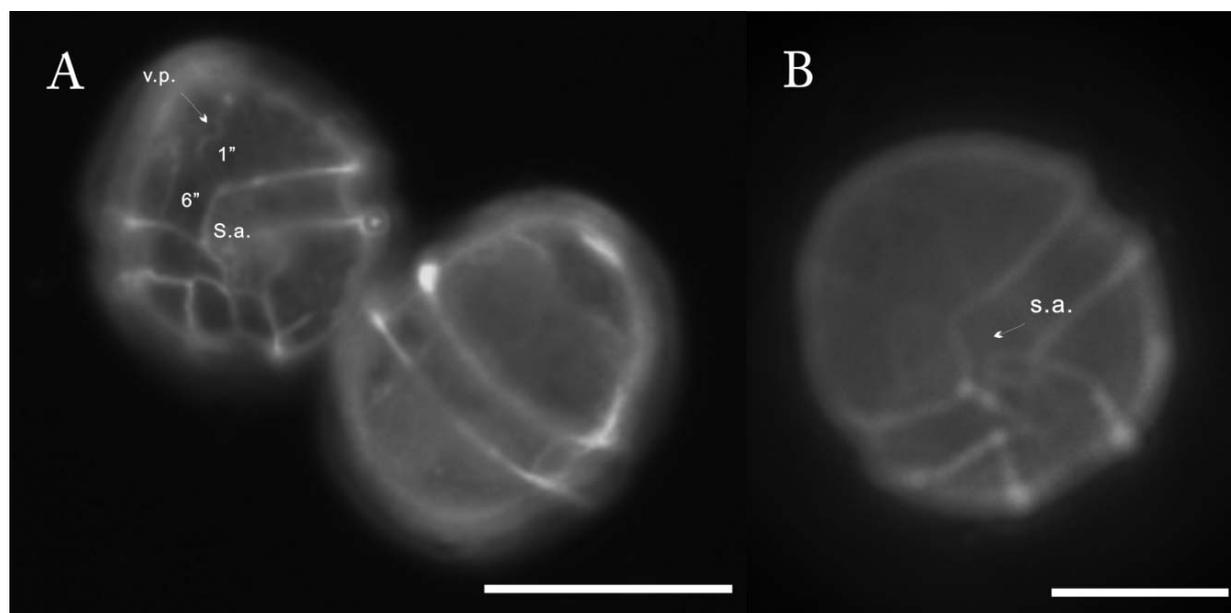
*In silico* probe design using ARB software was shown to be an advanced tool for probe design. Moreover, fluorescently labelled species-specific probe tested in this study showed high efficiency to rapidly detect cells of *A. minutum*, and did not show cross reactivity with other closely related species. Hence, the probe [L-S-Amin-569-A-18] shows great promise as a tool for rapid detection of *A. minutum* in Malaysia. In addition, optimization of the hybridization conditions has provided a better resolution of probe binding and efficiency.

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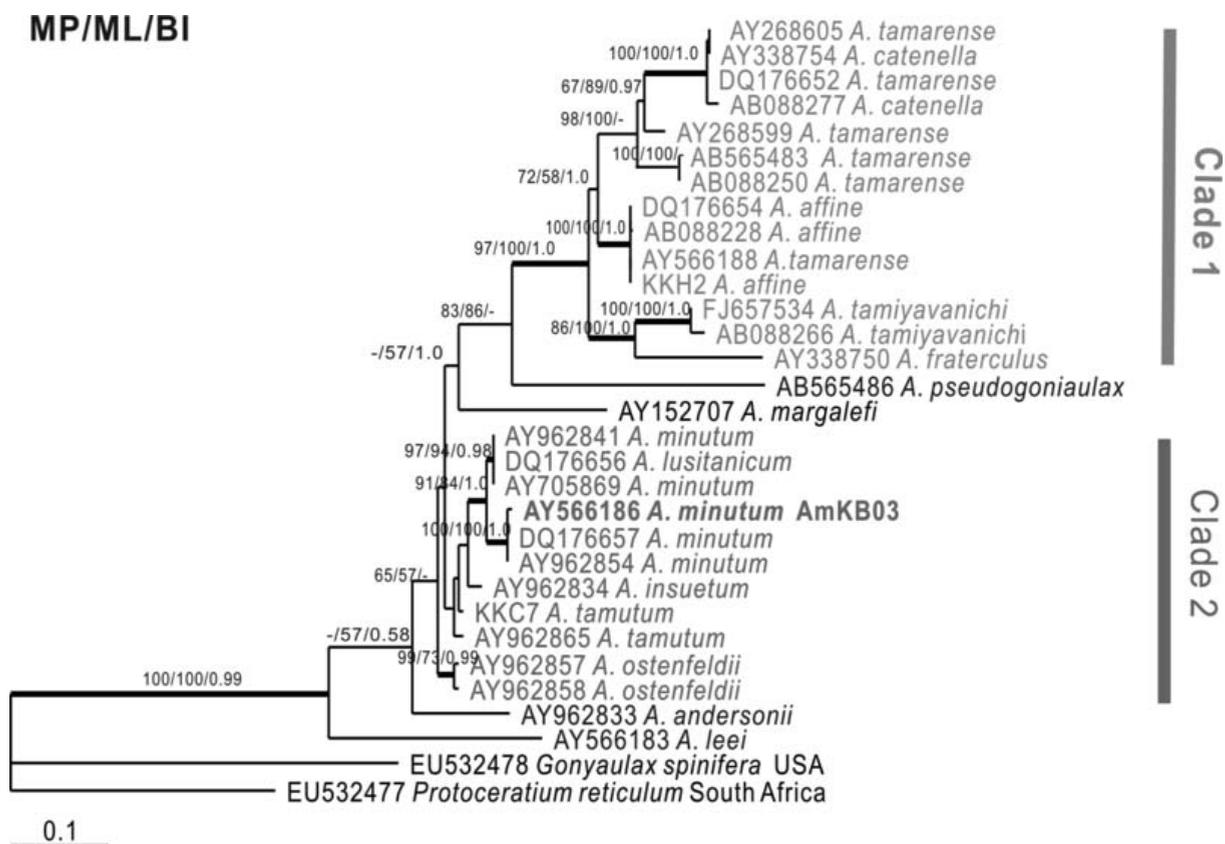
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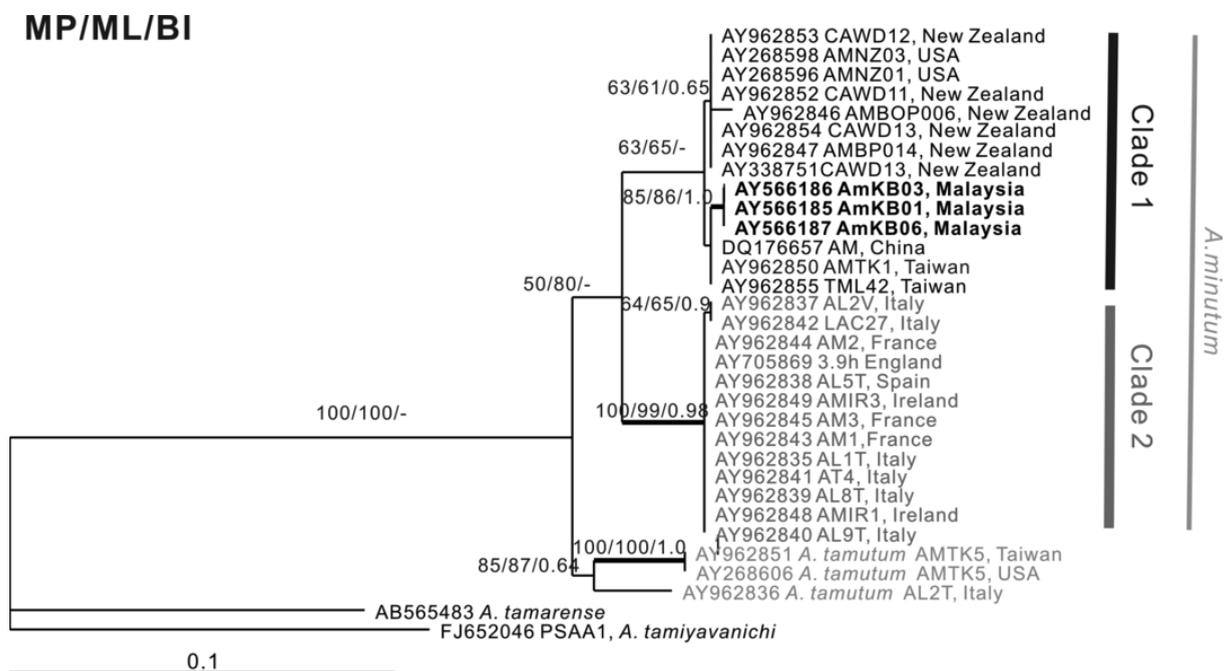
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**Figure 1.** Epi-fluorescence micrographs of cells of *A. minutum* and did (A) Ventral view showing first apical plate (1') with ventral pore (v.p.). The sixth precingular plate (6'') is narrow and long. (B) Anterior sulcal plate (s.a.). Scale bars = 10  $\mu$ m



**Figure 2.** Phylogenetic tree inferred from the sequences of LSU ribosomal RNA gene (D1-D2) of *Alexandrium* species. Scale bar represents 0.1 evolutionary rates.



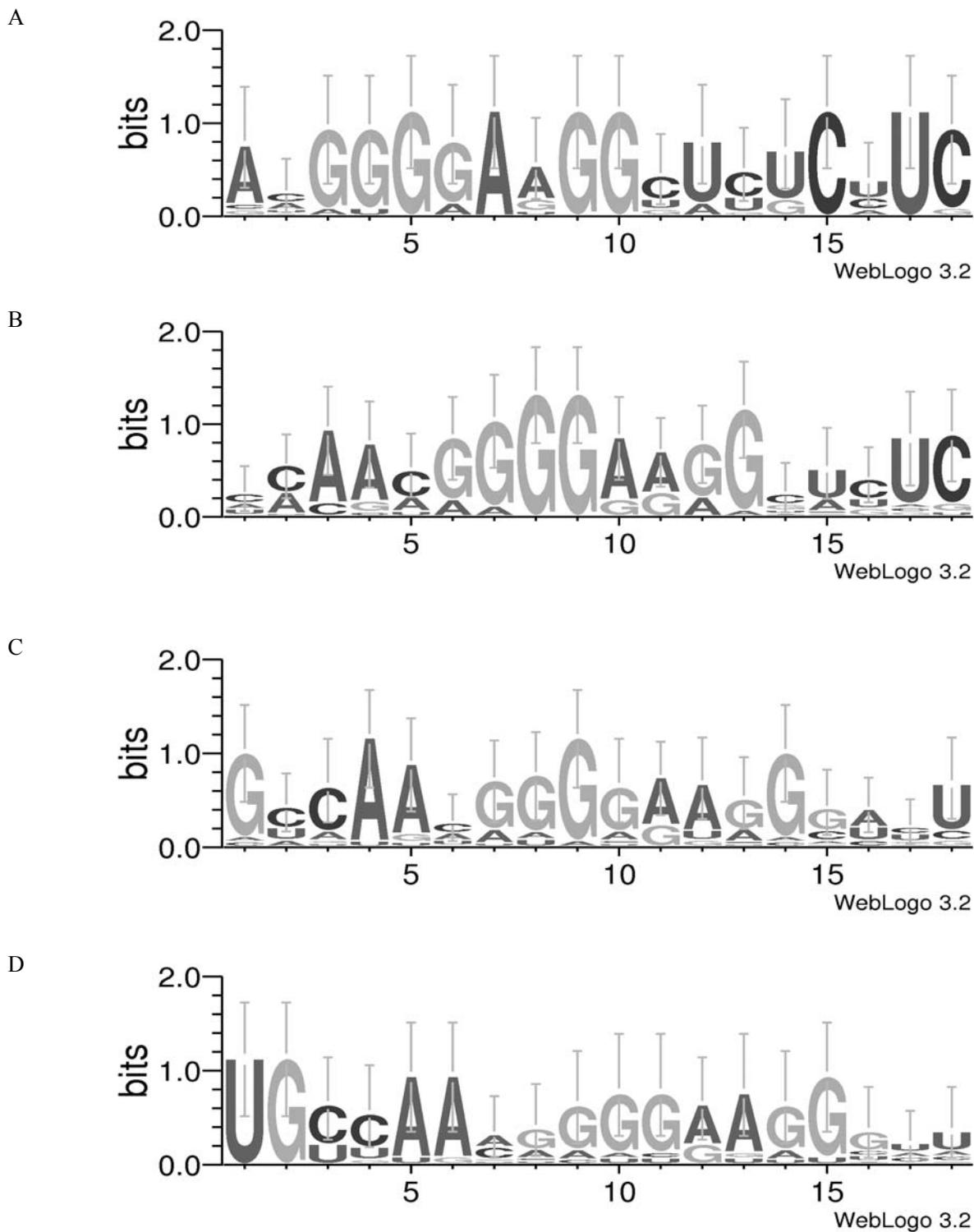
**Figure 3.** Phylogenetic tree inferred from the sequences of LSU ribosomal RNA gene (D1-D2) of *Alexandrium minutum*. Scale bar represents 0.1 evolutionary rates.

**Table 1.** Potential probe regions of *Alexandrium minutum* rDNA (D1-D3) based on ARB Probe Design Tool (PDT).

NO.	Probe sequence (5'-3')	$\Delta G^{\circ}_1$	$\Delta G^{\circ}_2$	$\Delta G^{\circ}_3$	$\Delta G^{\circ}_{overall}$	Hybridization Efficiency
1	AAGAGTCCCTTCCCCGTT	-23.4	0.6	-8.4	-14.7	0.9997
2	CTCAAGGAAGAGTCCCTT	-18.2	-0.3	-8.1	-9.4	0.4154
3	GAAGAGTCCCTTCCCCGT	-23.9	-0.4	-6.0	-17.1	1.0000
4	CAGTGCTCAAGGAAGAGT	-16.6	0.2	-7.6	-8.6	0.1523
5	GCTCAAGGAAGAGTCCCT	-19.9	-0.3	-8.1	-11.2	0.9157
6	TCCACCAACCACATGCAT	-19.7	1.6	-7.8	-11.9	0.9705
7	AGAGTCCCTTCCCCGTTG	-24.2	1.3	-8.4	-15.6	0.9999
8	AAGTCCACCAACCACATG	-17.8	1.7	-7.8	-9.9	0.5951
9	GAGTCCCTTCCCCGTTGG	-25.3	0.6	-8.4	-16.7	1.0000
10	GGAAGAGTCCCTTCCCCG	-23.8	-2.1	-6.0	-15.6	0.9999
11	TCCCCGTTGGCAAAACAG	-19.0	-0.1	-10.1	-8.4	0.1203
12	TCAAGGAAGAGTCCCTTC	-17.8	-0.7	-8.7	-8.2	0.0901
13	AGTGCTCAAGGAAGAGTC	-16.4	0.2	-8.1	-8.0	0.0642
14	CCACCAACCACATGCATC	-19.7	1.6	-7.8	-11.9	0.9705
15	AGTCCACCAACCACATGC	-20.4	1.6	-7.8	-12.6	0.9897
16	AGTCCCTTCCCCGTTGGC	-26.6	0.4	-9.5	-16.8	1.0000
17	CCCCGTTGGCAAAACAGC	-20.4	-0.1	-9.6	-10.3	0.7212
18	CAAGGAAGAGTCCCTTCC	-19.2	-2.3	-7.9	-8.9	0.2389
19	GTGCTCAAGGAAGAGTCC	-18.3	-0.1	-8.1	-9.7	0.5326
20	AAGGAAGAGTCCCTTCCC	-20.5	-2.6	-6.0	-11.8	0.9690
21	TGCTCAAGGAAGAGTCCC	-19.0	-0.3	-8.1	-10.2	0.7153
22	AGGAAGAGTCCCTTCCCC	-23.2	-2.6	-6.0	-14.6	0.9996
23	AAATGACAGAGTGGGCAC	-15.4	1.0	-19.5	4.1	3.6928e-10
24	CCGTTGGCAAAACAGCAC	-17.4	-0.1	-7.8	-9.1	0.2937
25	ACAAAGTCCACCAACCAC	-17.2	1.7	-6.3	-10.8	0.8661

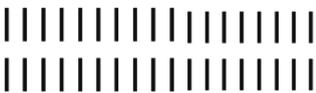
**Table 2.** Hybridization parameters obtained from ARB for the four potential probe regions.

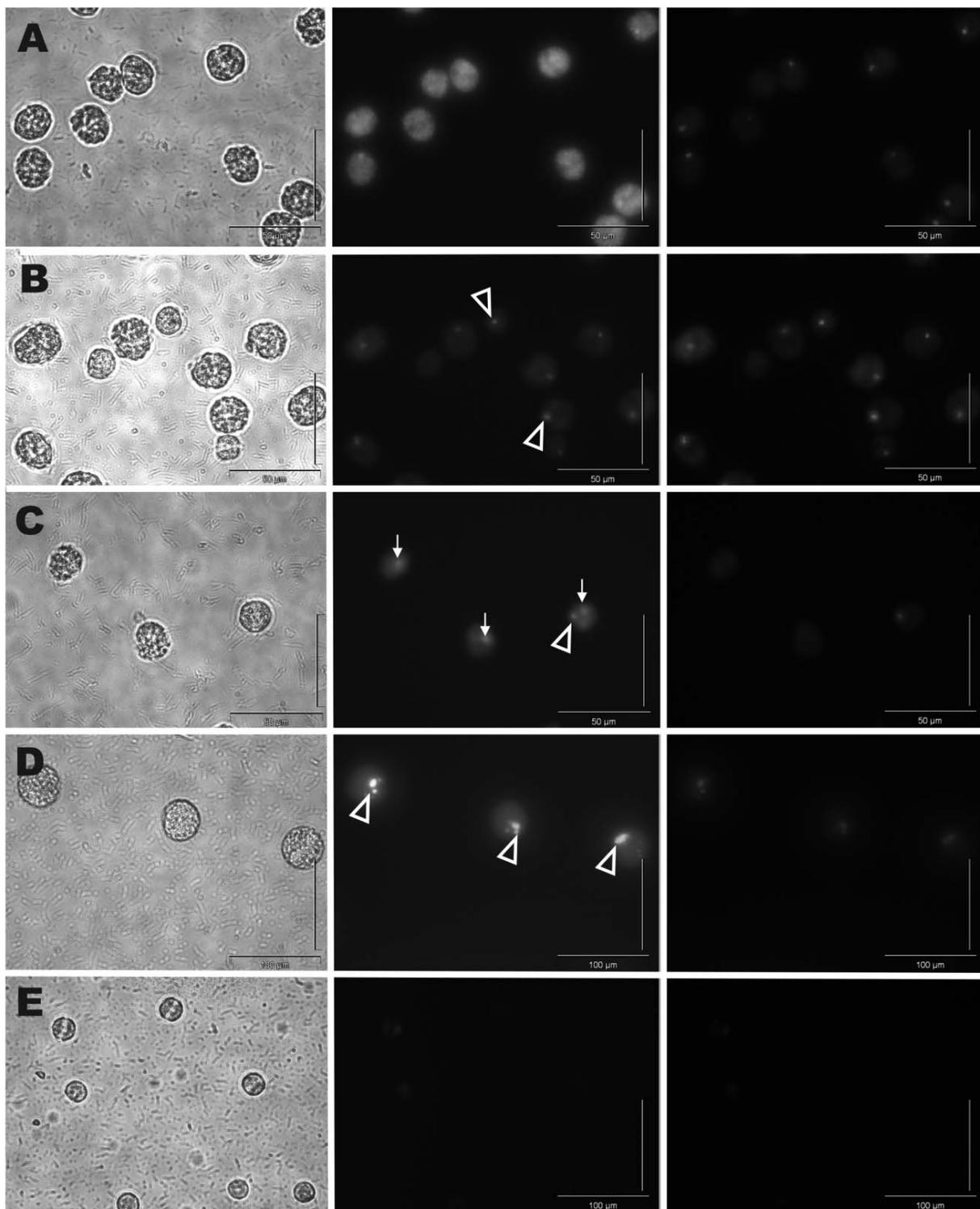
Probe	Target sequence (5' -3')	Probe sequence (5' -3')	Length of probe	G+C	4GC+ 2AT (melting temperature)
1	ACGGGGAAGGGACUCUUC	GAAGAGTCCCTTCCCCGT	18	61.1	58.0
2	CCAACGGGGAAGGGACUC	GAGTCCCTTCCCCGTTGG	18	66.7	60.0
3	GCCAACGGGGAAGGGACU	AGTCCCTTCCCCGTTGGC	18	66.7	60.0
4	UGCCAACGGGGAAGGGAC	GTCCCTTCCCCGTTGGCA	18	66.7	60.0



**Figure 4.** Signature regions of *Alexandrium minutum* LSU rDNAAs potential probe regions. (A) Probe 1, (B) Probe 2, (C) Probe 3, and (D) Probe 4.

**Table 3.** Mismatch analysis of probe region (Probe 3: [5'-AGUCCCUUCCCCGUUGGC-3']) against target species (species in Clade 1, Figure 3) and non-target species (species in Clade 2, Figure 3)

Non-target organism (Accession number)	Target and non-target organisms Species (strain/origin)	Complementary of probe towards orthologous target site	Number of mismatches
AY566187	<i>A. minutum</i> (AmKB06/ Malaysia)	Probe: 3'CGGTTGCCCTTCCCTGA5'  Target: 5'GCCAACGGGGAAGGGACT 3	0
AY962855	<i>A. minutum</i> (TML-42/ Taiwan)	Probe: 3'CGGTTGCCCTTCCCTGA5'  Target: 5'GCCAACGGGGAAGGGACT 3	0
AY962845	<i>A. minutum</i> (AM3/ France)	...G.....G...C....TT...C.....G... Probe: 3'.GTT.C.CC..C.CT.A5'  Target: 5'.CAA.G.GG..G.GA.T3' .....T.....T.A....GT..A..T...	7
	<i>A. affine</i> (KKH2)	...G.....G...C....TT...C.....G... Probe: 3'.GTT.C.CC..C.CT.A5'  Target: 5'.CAA.G.GG..G.GA.T3' ....T.....T..A....GT..A.....A...	7
	<i>A. tamutum</i> (KKC7)	...G.....G...C....TT...C.....G... Probe: 3'.GTT.C.CC..C.CT.A5'  Target: 5'.CAA.G.GG..G.GA.T3' ...T.....T..A....GT..A.....A...	7
AY438018.1	<i>A. hiranoi</i> (NIES-612)	... ..TG..C.....TT...CC...G.. Probe: 3'CGGT..C.CC..C..T.A5'  Target: 5'GCCA..G.GG..G..A.T3' .....TT..A....GT..AA....T...	8



**Figure 5.** Whole-cell FISH applied on *Alexandrium minutum*, *A. tamiyavanichii* and *A. tamutum* hybridized with uniC positive control (A), uniR negative control (B), species-specific probe towards *A. minutum*(C), *A. tamiyavanichii*(D) and *A. tamutum*(E). Arrows indicate green fluorescent signals from species-specific probe, and arrow heads mark the yellow fluorescent bodies in the cells. Micrographs of these species were taken under bright field (left), blue filter (center) and green filter (right), respectively.