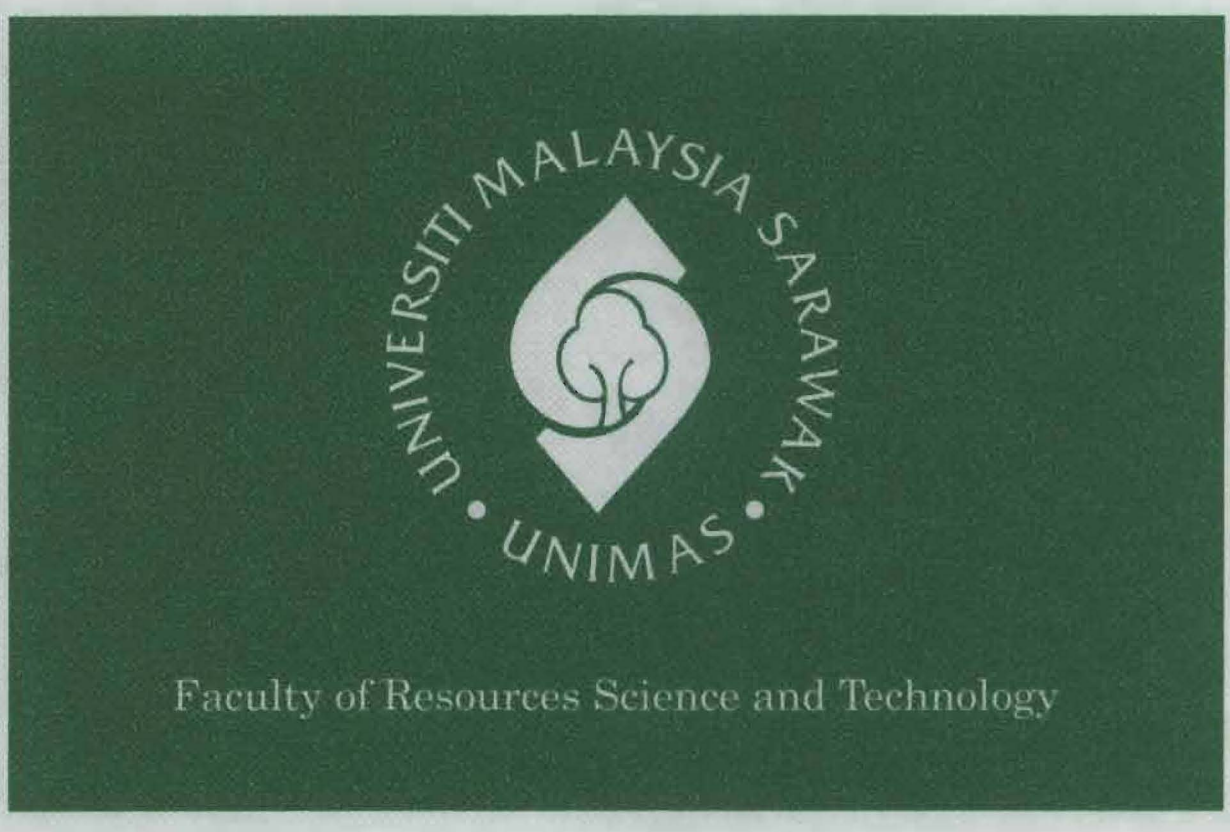

SOLVING THE PHYLOGENY OF TOXIC DINOFLAGELLATES, *ALEXANDRIUM* HALIM (DINOPHYCEAE)

Hii Kieng Soon

Bachelor of Science with Honours
(Resource Biotechnology)
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Solving the Phylogeny of Toxic Dinoflagellates, *Alexandrium* Halim (Dinophyceae)

Hii Kieng Soon

This project is submitted in partial fulfilment of the requirement of the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: Dr Leaw Chui Pin
Co-Supervisor: Dr Lim Po Teen

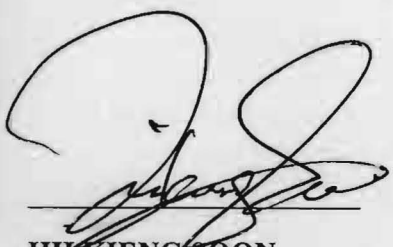
Resource Biotechnology
Molecular Biology

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

2010

Declaration

I hereby declare that the thesis is based on my original work except for quotation and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.



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LIST OF ABBREVIATIONS

APC	Apical Pore Complex
BMCMC	Bayesian Markov Chain Monte Carlo
CBC	Compensatory Base Changes
BLAST	*Basic Local Alignment Search Tool*
BI	Bayesian analysis
dNTP	Deoxyribonucleotide triphosphate
ETS	External Transcribed Sequence
ITS	Internal Transcribed Spacer
ITS1	First Internal Transcribed spacer
ITS2	Second Internal Transcribed spacer
LSU	Large Subunit
$MgCl_2$	Magnesium Chloride
PAUP	Phylogenetic Analysis Using Parsimony
PNJ	Profile Neighbour Joining
PSP	Paralytic Shellfish Poisoning
ML	Maximum Likelihood
MP	Maximum Parsimony
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
S.P	Sulcal Plate
STX	Saxitoxin
TBR	Tree Bisection-Reconnection

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Solving the Phylogeny of Toxic Dinoflagellates, *Alexandrium* Halim (Dinophyceae)

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ABSTRACT

The genus *Alexandrium* is a widely distributed dinoflagellate and has the ability to produce potent neurotoxins, saxitoxin (STX) and its deriavatives. In this study, the molecular phylogenetic approach was used to infer the relationships of *Alexandrium* species distributed worldwide. The clonal cultures of *Alexandrium minutum* (AmKB01, AmKB02, AmKB03, AmKB04, AmKB05 and AmKB06) was used in this study and maintained in SW II medium at 26°C under 12:12 hour light: dark cycle. The total genomic DNA of late exponential phase cultures was extracted and the genomic DNA were used for internal transcribed spacer (ITS) regions amplification and sequencing. Structural analysis of the ITS2 transcript was carried out using the sequences obtained together with related sequences from eighteen species of *Alexadrium, Pyrodinium bahamense* var. *compressum* and two outgroup taxa, *Coolia malayensis* and *Ostreopsis lenticularis*. The results showed conserved four helices secondary structure of ITS2 transcript for all sequences analyzed. Structural comparison between species found a number of compensatory base changes (CBCs). Structural information was then used to perform multiple sequence-structure alignment for phylogenetic analysis. The phylogenetic inference of *Alexandrium* species from this study showed nearly similar framework as in the LSU rDNA phylogeny.

Key words: *Alexadrium,* paralytic shellfish poisoning, ribosomal DNA, internal transcribed spacer regions, secondary structures modeling

ABSTRAK

Genus *Alexandrium* adalah Dinoflagelata yang mempunyai keupayaan untuk menghasilkan neurotoksin, saxitoksin (STX). Dalam kajian ini, filogenetik molekul digunakan untuk mengkaji hubungan spesis *Alexandrium* di seluruh dunia. Sampel kultur *Alexandrium minutum* (AmKB01, AmKB02, AmKB03, AmKB04, AmKB05 dan AmKB06) telah digunakan dalam kajian ini dan dikulturkan dalam media SW II pada suhu 26 ° C dalam kitaran cahaya 12:12 jam cahaya: gelap. DNA genomik pada kultur fasa eksponen akhir dipencil dan DNA digunakan untuk mengamplifikasikan kawasan transkripsi spacer dalaman (ITS) dan seterusnya penjujukan gen dijalankan. Analisis struktur transkrip ITS2 telah dijalankan dengan menggunakan jujukan yang diperolehi serta lapan belas jujukan spesis *Alexadrium* yang lain bersama *Pyrodinium bahamense var. compressum* dan dua taxa kumpulan luar, *Coolia malayensis* dan *Ostreopsis lenticularis.* Hasil kajian ini menunjukkan struktur sekunder transkrip ITS2 mempunyai empat heliks di kalangan spesis. Di samping itu, perbandingan struktur antara spesis menunjukkan wujudnya petukaran bes kompensatori (CBCs). Informasi struktur digunakan selanjutnya dalam penjajaran jujukan-struktur untuk analisis filogenetik. Keputusan kajian ini menunjukkan persamaan dari segi pokok filogeni yang dijana oleh data ITS2 *Alexandrium* dengan pokok filogeni yang dijanakan oleh jujukan rDNA LSU.

Kata kunci: *Alexadrium*, lumpuh keracunan kerang, ribosomal DNA, transkripsi spacer dalaman, permodelan struktur sekunder

1.0 INTRODUCTION

Alexandrium is a widely distributed dinoflagellate genus in many coastal regions around the world. Many species from this genus have the ability to produce potent neurotoxin, saxitoxin (STX) and its derivatives. The toxins cause paralytic shellfish poisoning (PSPs) through the consumption of shellfish which is contaminated by this toxins (Cembella, 1998). Proliferation of the species in the coastal region can cause serious economic and health problem (Medcof, 1985; Shumway and Cembella, 1993).

The genus *Alexandrium* consists of more than 30 species including the closely related toxic species such as *A. Catenella, A. tamarense* and *A. fundyense.* Taxonomists usually identified the dinoflagellate species by determining their fine-scale morphological features. The general cell form, shape of apical pore plate and present or absent of a ventral pore on the 1' apical plate were used primarily to classify these species (Taylor, 1984; Balech, 1985; Fukuyo, 1985). However, the phenotypic characters expression may vary in response to changing environmental condition as well as growth stage (Sako et al., 1990). In addition, the criteria used to classified these species are different depend on taxonomist (Taylor, 1984; Balech, 1985; Fukuyo, 1985; Cembella and Taylor, 1986; Steidinger and Moestrup, 1990).

In the last two decades, taxonomic ambiguities in the species have been addressed and different approaches have been proposed to solve the taxonomic confusion in the genus. Molecular phylogenetic approach became one of the most widely accepted approaches to support the taxonomy of this genus. The nuclear-encoded large subunit of

ribosomal DNA (LSU rDNA) was commonly used in the phylogenetic analysis to classify the taxonomy of the species (Scholin *et al.*, 1996; Yeung *et al.*, 2002; Lily *et al.*, 2007). However these studies showed intra- and inter-specific uncertainty in delineating the species. The taxonomy of the species complex such as *A. tamarense*, *A. fundyense*, and *A. catenella* which are together comprise as a *A. tamarense* species complex still cannot be solved by LSU rDNA and morphology phylogenetic analysis.

The internal transcribe spacer (ITS) region have been considered as a promissory nuclear region in molecular phylogenetic at intra-genus level (Adachi *et al.*, 1996). However high degree of intra-variability in the sequences of ITS regions due to excess INDEL (insertion-deletion) made sequence alignment difficult and phylogenetic analysis noisy. Recently the ITS2 region has been found to provide very useful biological information. Muller *et al.* (2007) and many others (e.g. Amato *et al.*, 2007; Coleman, 2009) showed that the secondary structure of ITS2 formed by the initial RNA transcript could be used to characterize members of a species.

The main goal of this study was to review the phylogenetic relationship and to solve the phylogenetic confusions in the genus *Alexandrium*. In this study, the nucleotide sequences of the ITS regions of the nuclear encoded ribosomal RNA gene of *Alexandrium* species from Malaysian waters were obtained by sequencing. The ITS2 sequences were used to model the secondary structure of ITS2 transcript. The structural information of the unambiguous sequence-structure alignment of ITS2 was used to reconstruct a reliable phylogenetic framework to infer the phylogenetic lineage of the species in the genus.

2.0 LITERATURE REVIEW

2.1 Research Background of *Alexandrium* species

The *Alexadrium* species have the ability to produce neurotoxin which is saxitoxin (STX). This neurotoxin will contaminate the shellfish and will cause paralytic shellfish poisoning (PSP) when the shellfish is consuming (Cembella, 1998). Doucette *et al.*, (1997) figured out the STX will bind to the voltage dependent sodium channel and will inhibit channel open. In neurotransmission, the voltage dependent sodium channel plays an important role at the neuronal synapses and neuromuscular. The binding of STX on the voltage dependent sodium channel will inhibit the nerve conduction and will cause the victim tingling sensations, headaches, fever, rash, dizziness, gastrointestinal illness, muscular paralysis, pronounced respiratory difficulty, and choking sensation. However, about one to four milligram of the STX will become lethal and cause respiratory paralysis and loss motor control (Evans, 1972). The *Alexandrium* species which have the ability to produce STX included *A. caternella, A. fundyense, A. minutum,* and *A. tamarense.*

The morphological characteristics such as thecal plate and the position of the plates have been used as the tool to classify the taxonomy of this species (Balech, 1989; 1995). For example, Leaw *et al.* (2005) investigated the diagnostic morphological features of Malaysian *Alexandrium* and *Pyrodinium bahamense* to study their taxonomy (Figure 2.1). This combined morphological-molecular approach of phylogenetic analyses used 16 morphological characters to infer the character evolution of the species (Figure 2.2). The total evident approach adopted in the study yielded the same results of the large subunit ribosomal RNA gene (LSU rDNA) phylogenetic analysis.

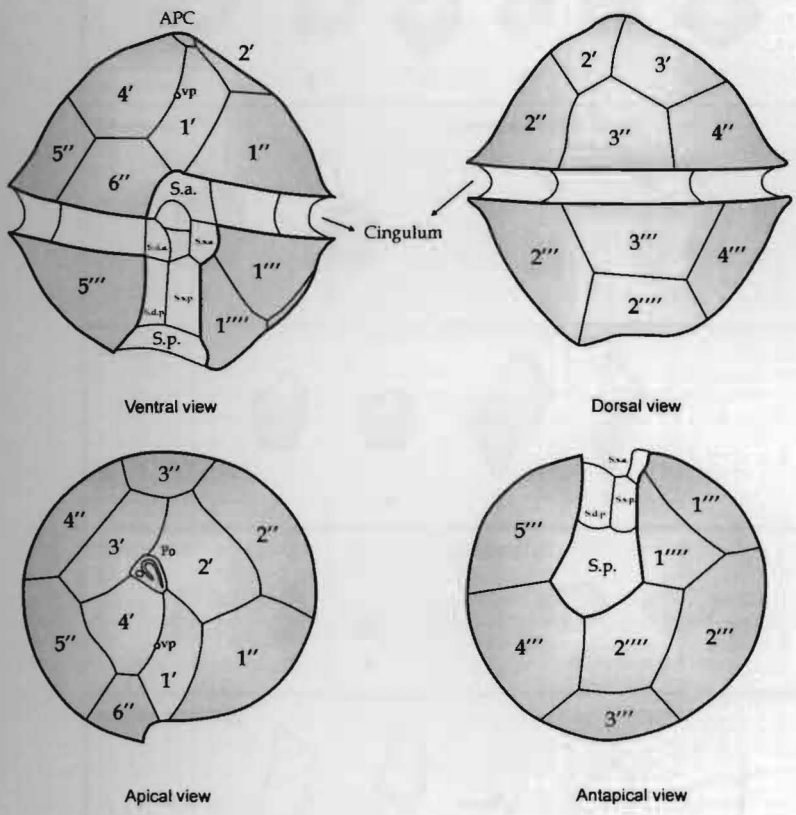

Figure 2.1: Theca plate tabulation of *Alexandrium* species showing the ventral, dorsal, apical and antapical views. Apical plates are represented as (′), precingular plates as (″), postcingular plates (‴) and antapical plates (⁗) (adopted from Leaw *et al.*, 2005).

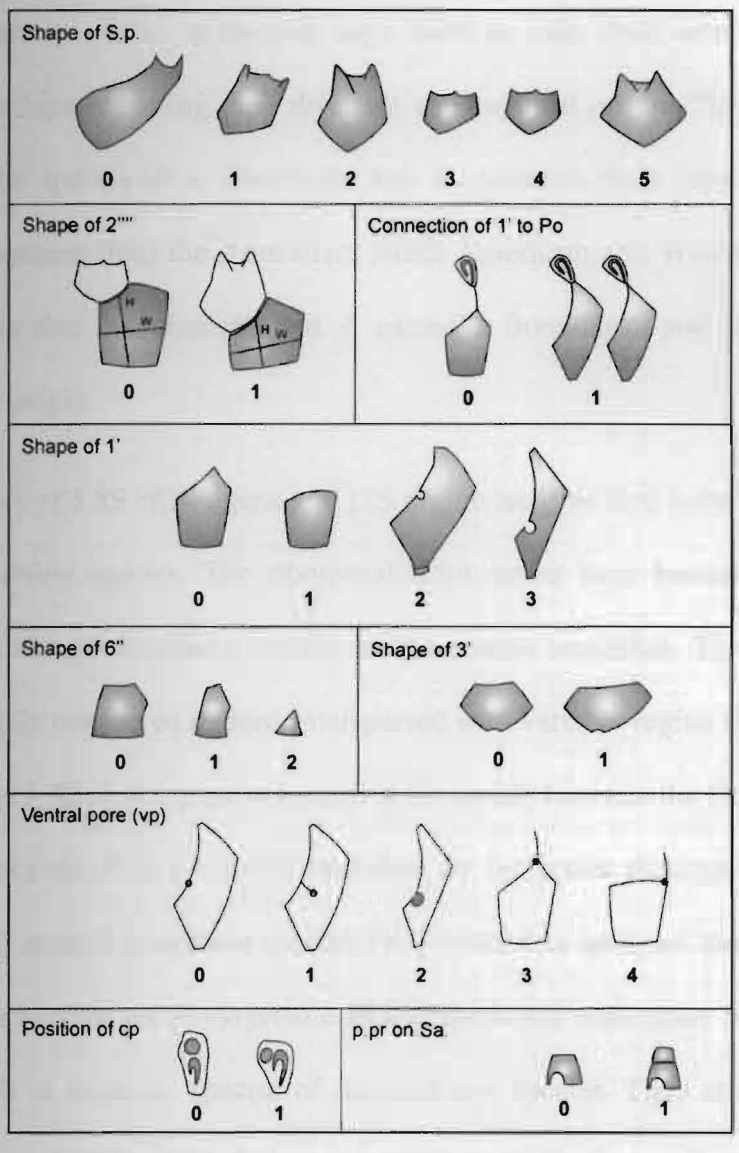

Figure 2.2: Schematic drawing of the morphological character analyzed (adopted from Leaw *et al.*, 2005).

The phylogenetic analysis of the LSU rRNA gene has been used to reveal the taxonomy of these *Alexamdrium* species. The region commonly used is the LSU rDNA containing D1 and D2 hypervaribble domains. The acceleration rate of nucleotide substitution in LSU rRNA sequence is suitable used as a tool to infer the species taxonomy. For example, previous study done by Scholin *et al.* (1994) showed that the sequences of the LSU rRNA gene of *A. tamarense, A. catenella, A. fundyense, A. affine, A.*

minutum, A. lusitanicum and A. andersoni were used to infer their interspecific and intraspecific relationships regarding their different geographical origin. The LSU rRNA gene showed that the species of *A. tamarense* and *A. catenella* from Japan were most probably the same species from the Australian, North American, and Western European strains. Besides, they also hypothesized that *A. catenella* from Japan and Australia was come from the same origin.

The sequences of 5.8S rRNA gene and ITS region analysis also have been used to identify the *Alexandrium* species. The ribosomal RNA genes have become the useful choice in the molecular phylogenetic studies on taxonomic evolution. This is because rDNA consist of highly conserved regions interspersed with variable region (Medlin *et al.*, 1988). However, the 5.8S rRNA gene is located at the center between the ITS1 and ITS2 of the rDNA sequence which is a suitable candidate for molecular phylogenetic studies. Adachi *et al.* (1996) applied neighbour joining (NJ) method to analyses the 5.8S rRNA gene and ITS region to infer the phylogenetic relationships and population boundaries of toxic species as well as nontoxic species of *Alexandrium* species. They showed that *A. tamarense* from Japan and the United States as well as *A. fundyense* from the United States claded together and were closely related to each others. In contrast, *A. tamarense* from Thailand showed high heterogeneity in the ITS regions even though they were morphologically indistinguishable (Adachi *et al.*, 1996).

2.2 Species complex and the taxonomic uncertainty in *Alexandrium* species

The species complex which comprised of *A. tamarense*, *A. catenella* and *A. fundyense* have caused a lot of confusion to the species identification in the genus. Many researchers contended and believed that the three morphospecies (*A. tamarense*, *A. catenella* and *A. fundyense)* which can be defined through the morphology are probably same biological

species. However, Scholin *et al.* (1995) contended that the morphological characteristics cannot be used to identify the species. The genetic heritage may be varied within the species, although the morphospecies are highly similar in overall appearance, distinguished mainly by chain-forming ability, cell shape, and the ventral pore between precingular plates of 1′ and 4′ (Balech, 1995).

However, the ability to produce saxitoxins among this three *Alexandrium* species are different. *A. tamarense* strains can be either toxic or nontoxic but all the *A. catenella* and *A. fundyense* are toxic species. Some studies on inbreeding experiments of the three species have shown that they sexually incapability and thus are not same biological species (Sako *et al.*, 1990; Mackenzie *et al.*, 2004).

In the study of Usup *et al.* (2002a), *A. tamiyayanichii* also grouped together with the *A. tamarense* species complex. The 28S, 18S and 5.8S subunit of rRNA gene sequence analyses showed similar topology. The 5.8S subunit also showed that *A. tamiyavanichii* grouped together with *A. fundyense* and *A. tamarense*. However, in the study of Lilly *et al.* (2007), *A. tamiyayanichii* was clustered out from the *A. tamarense* species complex. This has caused some confusion in the taxonomy of this species complex by using rRNA gene analysis.

2.3 The second internal transcribed spacer (ITS2) transcirpt

The internal transcribed spacer (ITS) is a non-functional RNA which is positioned between structural ribosomal RNAs (rRNAs) on a polycistronic precursor transcript. This polycistronic rRNA precursor transcript have 5' external transcribed spacer (5' ETS), 18S rRNA, ITS 1, 5.85S rRNA, ITS2, 28S rRNA and 3' external transcribed spacer (3' ETS) (Figure 2.3).

Figure 2.3: The single repeat of ribosomal RNA gene precursor. The ITS2 is located between the 5.8S rRNA gene and the large subunit (LSU) RNA gene (28S) (adopted from Cooke and Duncan, 1997).

The ITS2 region is situated between the 5.8S rRNA gene and the large subunit (LSU) RNA gene which also known as 28S rRNA in a polycistronic precursor transcript. The ITS1, ITS2 and ETS sequence will be degraded when the polycitronic precursor were transcribed into the mature rRNA. The ITS1, ITS2 and ETS will become non-functional sequence when the rRNA is mature. However, Mai and Coleman (1997) pointed out that the secondary structure of the ITS2 transcript could be served as a useful taxonomic tool in species classification.

Recently, the ITS2 secondary structure of all eukaryotes has been shown to have the same four helix structure characteristics which were observed from the high resolution transmission electron microscope (TEM) (Coleman 2003, 2007; Muller *et al.*, 2007). In the ITS2 transcript secondary structure, the first and fourth helices are useful tool for species and subspecies comparisons(Figure 2.4). This is because the first and four helices are the most evolutionary variable. Moreover, the second and third helices of ITS2 structures among all eukaryote are constant. The second and third helices are much preservable than the first and fourth helices. These constant features showed that both of the second and third helices contain molecular signal to succeed the RNA transcription process. The characteristic of second helix is rather short and the vicinity of the base

containing a pyrimidine-pyrimidine mismatch on it. However, the third helix is longer than other helices and even has a branch on the 5'-side near the tip. This single branch on the third helix is the most preserved stretch of nucleotides in the entire ITS2 region (Coleman, 2009). Cote *et al.* (2002) observed that, in the process of ITS2 RNA transcript, there will be one or two initial cleavages in the region extending along 3' side of second helix to 5' side of helix III (Cote *et al.*, 2002).

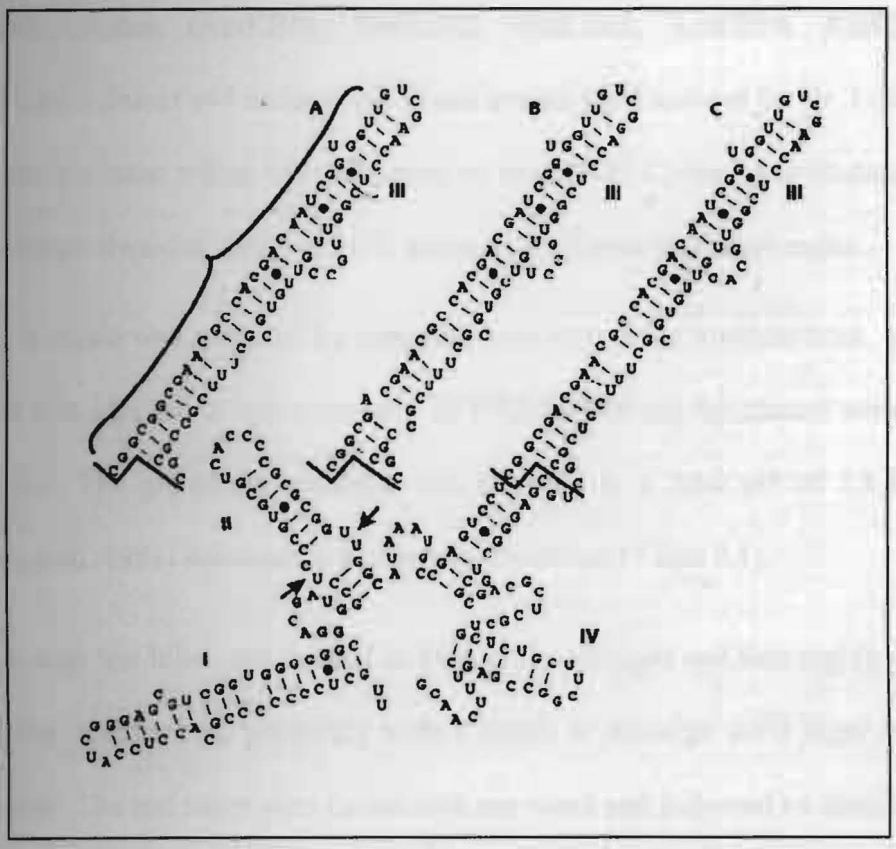

Figure 2.4: The secondary structure of folding ITS2 with four-helix structure characteristic (adopted from Coleman, 2009).

3.0 MATERIALS AND METHODS

3.1 Algal cultures

Clonal culture of *Alexandrium* species used in this study was obtained from the UNIMAS phytoplankton culture collection in the Laboratory of Eco-toxicology. Six strain of *Alexandrium minutum* (AmKB01, AmKB02, AmKB03, AmKB04, AmKB05 and AmKB06) were cultured and maintained. These strains were isolated by Dr. Lim Po Teen from Tumpat, Kelantan which were bloomed on year 2000. Culture was routinely grown in SW II medium (Iwasaki, 1961) at 26°C under a 12:12 hour light:dark cycle.

The medium was prepared by using the seawater as the medium base. Salinity of the medium was adjusted to approximately 15 PSU by diluting the natural seawater with distilled water. The pH of the seawater was adjusted to a final pH of 7.8-8.2. SWII medium (Iwasaki, 1961) was used as the enriched medium (Table 3.1).

The clean test tubes was soaked in 10% HCl overnight and thoroughly washed in phosphate-free detergent by scrubbing with a brush to dislodge solid algal remains or other materials. The test tubes were rinsed with tap water and followed by distiller water.

Approximately 25-mL of medium was transferred into the cleaned test tube and sterilization was done by autoclaving at 121°C for 15 minutes. After autoclaving, media were left about 24 hours before used to allow the CO_2 gases to diffuse into the liquid.

Table 3.1: The SWII medium condition (Iwasaki, 1961)

	Stock concentration	Volume added into 1L of seawater (mL)	Final Concentration
KNO_3	7.2×10^{-3} mol/L	1.0	7.2×10^{-4} mol/L
KH_2PO_4	3.31×10^{-4} mol/L	1.0	3.31×10^{-5} mol/L
Na_2-glycero. PO_4	3.33×10^{-4} mol/L	1.0	3.33×10^{-5} mol/L
Vitamin Mix		1.0	
➢ Vitamin B_{12} (cyanocobalamin)			4.43×10^{-10} mol/L
➢ Biotin			4.1×10^{-9} mol/L
➢ Thiamine-HCl			3×10^{-7} mol/L
Fe-EDTA		1.0	1.19×10^{-6} mol/L
Tris-HCl (pH 7.8)		1.0	4.13×10^{-3} mol/L

3.2 Genomic DNA extraction

Approximately 25 ml of exponential phase culture was transferred into a 50 ml centrifuge tube and harvested by centrifugation at 2000 ×*g* for 5 minutes. The cell pellet was then transferred into a 1.5 mL microfuge tube and cell lysis was carried out with 2× cetyltrimetylammonium bromide (CTAB) buffer containing 20 mM ethylenediamine-tetraacetic acid (EDTA), pH 8, 10 mM Tris-Base, pH 7.5, 50 mM CTAB, 14 mM NaCl and 0.2 M 2-*β*-mecaptethanol. A total of 5µl Proteinase K (20mg/nl) were added to the mixture, and the sample was incubated in water bath at 60 °C for 45 to 60 minutes.

After incubation, the cell was extracted once with chloroform: isoamyl alcohol (24: 1), once with phenol: chloroform: isoamyl alcohol (25: 24: 1) and ended with once chloroform: isoamyl alcohol (24: 1) again. The DNA will be precipitated by adding two volume of ice-cold ethanol (EtOH) and 1/10 volume of 3 M sodium acetate, pH 5. Then the sample will be kept at -20°C for 2 hours. The mixture will be centrifuged at 32000 ×*g* for 10 minutes and DNA pellet will be rinsed once with 70% EtOH, followed by centrifugation at 32000 ×*g*. The DNA pellet will then be air dried and resuspended with

TE buffer (10 mM Tris-HCl, pH 7.4 and 1mM EDTA, pH 8). The genomic DNA will be stored at - 20°C for further analysis. The quality and quantity of the DNA were determined by using Biochrom UV spectrophotometer (Libras 12, Biochrom, England).

3.3 DNA amplification, purification and sequencing

The ITS region of nuclear encoded ribosomal RNA gene was amplified by using a set of primers as described by White *et al.* (1990), ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC -3') The primers were synthesized by Vivantis (Vivantis Co., Malaysia).

The amplification was carried out in a 25 µL reaction mixture. The PCR cocktail containing 1× PCR buffer (Fermentas, USA), 2 mM $MgCl_2$ (Fermentas, USA), 0.2 mM of deoxynucleoside triphosphate (dNTP) (Fermentas, USA), 1 µM of each primer, 1 unit of *Taq* polymerase (Fermentas, USA) and 100 – 200 ng of template genomic DNA (Table 3.2). PCR amplification was performed in 35 cycles by using Eppendorf Gradient thermocycler (Eppendort, Germany). The PCR running condition was as detailed in Table 3.3.

The amplified products were visualized together with a Fermentas 100 bp DNA ladder on 1% agarose gel, run for approximately 1 hour at 90V. The gel was post-stained with 10 mg/mL ethidium bromide (EtBr), viewed under a UV-illuminator (Uvitec, EEC) and gel images were captured.

Table 3.2: PCR Cocktail Ingredient

PCR reagent	Stock	Final Concentration	Volume used in 25 reaction (μL)
Dream Taq™ Buffer +$MgCl_2$ (Fermentas, USA)	10 × Taq Buffer + 20mM $MgCl_2$	1 × Taq Buffer + 2mM $MgCl_2$	2.5
ITS 1 primer	25 μM	1 μL	1
ITS 4 primer	25 μM	1μL	1
dNTP (Fermentas, USA)	25 mM	0.2 mM	0.2
Taq (Fermentas, USA)	500 (5unit/μl)	1 unit	0.2
ddH_2O	-	-	19.1
DNA Template	-	(100-200ng)	1
		Total	25

Table 3.3: Reaction parameters for ITS region amplification

Cycle Step	Temperature	Duration Time
Initial denaturation	94°C	5 min
Denaturation	94°C	30 Sec
Annealing	51°C	30 Sec
Elongation	72°C	1 min
Final Elongation	72°C	7 min
Hold	4°C	∞

Purification of amplified products was performed using Promega purification kit, Wizard® SV Gel and PCR Clean-up system kit (Promega, USA) following manufacturer's instruction. The purified products were sent to a private sequencing laboratory (1st Base, Selangor, Malaysia) for DNA sequencing.