Faculty of Resource Science and Technology

MOLECULAR CHARACTERIZATION OF DIATOM, *PSEUDO-NITZSCHIA* SPECIES (BACILLARIOPHYCEAE) BASED ON SECONDARY STRUCTURES MODELING OF SECOND INTERNAL TRANSCRIBED SPACER (ITS2) TRANSCRIPT

TENG SING TUNG (20122)

Bachelor of Science with Honours
Resource Biotechnology
Molecular Biology
2010

Molecular characterization of diatom, *Pseudo-nitzschia* species (Bacillariophyceae) based on secondary structures modeling of second internal transcribed spacer (ITS2) transcript

TENG SING TUNG (20122)

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.

_____

TENG SING TUNG

Resource Biotechnology Programme,

Faculty of Resource Science and Technology,

University Malaysisa Sarawak.

ACKNOWLEDGEMENTS

Above all, I would like to thank my supervisor, Dr Leaw Chui Pin and co-supervisor, Dr Lim Po Teen for giving me an opportunity to work on this project under their guidance, valuable advice, generous suggestions and encouragement.

Besides that, I would like to thank all the seniors in the lab, Kak Tina, Hong Chang, Kak Su, Toh Hii, Kak Zu and Kak Fareha for sharing their experiences and knowledge with me. And also, I am grateful to the lab assistants, Kak Rahah and Abang Hasri for their assistances especially in term of technical problems. My lab mates, Kieng Soon and Bebe, fellow friends especially my course mates, thank you for your supports, friendship and advices. Lastly, my sincere gratitude is due to my family, my beloved parents and siblings who always encourage me and support me through my life.

This project was supported by the International Science Foundation (IFS) to Dr. Leaw.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ASP	Amnesic Shellfish Poisoning
C-TAB	Cetyltrimethylammonium Bromide
DA	Domoic Acid
DNA	Deoxyribonucleic acid
HABs	Harmful Algal Blooms
HMMs	Hidden Markov Models
ITS	Internal Transcribed Spacer
ITS1	First Internal Transcribed Spacer
ITS2	Second Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
Pm	Pulau Mamutik
Pn	*Pseudo-Nitzschia*
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
Sb	Santubong
SEM	Scanning electron microscopy
Sm	Semariang
SWII	Seawater II medium
PNJ	Profile-neighbour joining
MP	Maximum parsimorny
ML	Maximum likelihood
CBCs	Complementary Base Changes

LIST OF FIGURES

LIST OF TABLES

Molecular characterization of diatom, *Pseudo-nitzschia* species (Bacillariophyceae) based on secondary structures modeling of second internal transcribed spacer (ITS2) transcript

ABSTRACT

Diatoms in the genus *Pseudo-nitzschia* have been known as the causative organism in amnesic shellfish poisoning (ASP) events. Ten out of nearly 30 species thus far described have the ability to produce the potent neurotoxin, domoic acid. In Malaysia, however, has little attention paid to this form of shellfish poisoning. In this study, homologous modeling and free energy minimization approaches were used to model the secondary structure of the second internal transcribed spacer (ITS2) of *Pseudo-nitzschia* species. A total of 18 strains of *Pseudo-nitzschia* spp. from Borneo were cultured and genomic extracted. Gene amplification and sequencing of the ITS region of ribosomal RNA genes was carried out. Sequences obtained and related sequences were used to construct the secondary structure of ITS2 transcript. The results showed that the secondary structure predicted from *Psedudo-nitzshia* spp. consist four main paired helices with one pseudo-helix. Three motifs were found in the transcript. The common eukaryotic motif in the right of helix II with additional AAA was also found in all the structures. The structural analysis also showed conserved 5.8S-28S complex. The structural information was incorporated into the multiple sequence-structure alignment for phylogenetic analysis. The phylogenetic trees revealed two monophyletic clades. This was well supported by the morphological classification and the large subunit phylogentic evidences. Placement of some *Pseudo-nitzschia* spp. however remained unresolved. The ITS2 secondary structures information in this study provided better understanding of the *Pseudo-nitzschia* taxonomy and could be potential molecular tools in strain or species identification.

Key words: *Pseudo-nitzschia*; Amnesic Shellfish Poisoning (ASP); Internal Transcribed Spacer (ITS) region; ITS2 transcript

ABSTRAK

Diatom dalam genus Pseudo-nitzschia telah dikenali sebagai penyebab dalam peristiwa-peristiwa keracunan kerang-kerangan amnesik (ASP). Setakat ini, sepuluh daripada hampir 30 spesis Pseudo-nitzschia dibukti mempunyai keupayaan menghasil neurotoksin iaitu domoic asid. Walau bagaimanapun, hanya sedikit perhatian diberi kepada keracunan ini di Malaysia. Dalam kajian ini, kaedah homologi pemodelan struktur dan kaedah tenaga bebas perminiman digunakan untuk memodelkan struktur sekunder transkripsi dalaman kedua (ITS2) spesis-spesis Pseudo-nitzschia. Sejumlah 18 strain Pseudo-nitzschia dari Borneo telah dikultur dan genomik dipencil. Amplifikasi gen dan penjujukan kawasan transkripsi dalaman daripada gen ribosomal RNA telah dijalankan. Jujukan-jujukan yang diperolehi telah digunakan untuk menjana struktur sekunder transkrip ITS2. Hasil daripada kajian ini menunjukkan struktur-struktur Psedudo-nitzshia spp. mengandungi empat heliks utama dan satu palsu heliks. Tiga motif telah dijumpai dalam transkrip ITS2. Motif eukariotik juga dijumpai pada sebelah kanan helik II dengan AAA. Struktur sekunder yang dimodelkan untuk 5.8S-28S kompleks juga menunjukkan pemuliharaan yang tinggi. Informasi struktur telah digunakan dalam penyusunan jujukan-struktur yang seterusnya diguna dalam analisis filogenetik.Keputusan daripada pokok-pokok filogenetik mengandungi dua kumpulan monofiletik yang disokong oleh bukti-bukti dari kelasifikasi morfologi dan analisis filogenetik gen ribosomal subunit besar. Walau bagaimanapun,kedudukan sesetengah Pseudo-nitzschia spp.dalam pokok filogenetik masih diragu-ragui. Struktur sekunder ITS2 dalam kajian ini telah menyumbangkan pegetahuan dalam taksonomi Pseudo-nitzschia dan kemungkinan boleh diguna dalam pengecaman spesis dan strain secara teknik molekul.

Kata kunci: Pseudo-nitzschia; keracunan Kerang-kerangan amnesik (ASP); kawasan transkripsi dalaman; transkrip ITS2

1.0 INTRODUCTION

There are more than 200 genera of living diatoms with approximately 100,000 extant species. Some of the species are harmful and toxic to the marine life. Human being also affected by the intoxication due to the harmful algal blooms (HABs).

Amnesic Shellfish Poisoning (ASP) is a type of shellfish poisoning in human that caused by the diatom, *Pseudo-nitzschia*. In 1987, the first ASP case was found at the eastern Canada (Perl et al., 1990). This type of poisoning is caused by the neurotoxin, domoic acid (DA). DA is a neurotoxin which causes the damage of hippocampus and amygdaloid nucleus by activating AMPA and kainate receptors. This phenomenon causes an influx of calcium. In the ASP cases, victim shows the gastrointestinal symptoms like vomiting, nausea, diarrhea, abdominal cramps and haemorrhagic gastritis. In other cases, neurological symptoms also diagnosed, this include headache, dizziness, disorientation, vision disturbances, and loss of short-term memory, motor weakness, seizures, profuse respiratory secretions, hiccoughs, unstable blood pressure, cardiac arrhythmia, and coma. Besides that, domic acid is stable in extreme conditions, for example domoic acid is stable under cooking and freezing temperature.

In *Pseudo-nitzschia* taxonomy, species was only discernable by detailed morphological characteristics. There are valve length and width, fibula, stria and poroid densities, number of sector within paroids (Hasle, 1965; Hasle, 1994). However morphological observation on fine structures of the species often rely on the advanced electron microscopy (EM) which could not be accomplished by normal compound microscopy (Hasle & Syvertsen, 1997). Difficulties in identifying the fine structures of *Pseudo-nitzschia* made it difficult to detect and define them in nature. Sexual

compatibility characterization is one of the approaches that designed to characterize the species of *Pseudo-nitzschia* (Davidovich & Bates, 1998; Amato et al., 2005). But, somehow the taxonomy of *Pseudo-nitzschia* remained unresolved. This is because almost all *Pseudo-nitzschia* spp. have heterothallic mating system (Amato et al., 2005).

In delineating the diatom species concept, phycologists commonly delineate species based on the descriptions sorely upon morphological characterization. However, this conventional approach has been debated for decades and the morphological species concept has been questioned. This has given rise to the second approach of recognition of the biological species concept where individuals capable of interbreeding are each others' nearest relatives (Dobzhansky, 1940). Thus, secondary characterization of species have been introduced and applied in delineating the species. Among other characterizations, such as sexual compatibility characterization (Amato, 2007), molecular characterizations (ribosomal genes) become popular and most widely used in defining the species level of studied organisms.

Molecular characterization using the internal transcribed spacer (ITS) region analysis is one of the recent approaches that have been applied to characterize the species of *Pseudo-nitzschia* (Amato et al., 2007). The objective of this study is to characterize the species of *Pseudo-nitzschia* that were found in the Sarawak waters by using the structural information of the secondary structure of ITS2 transcript. In this study, the ITS region of the ribosomal RNA genes of the Sarawakian strains of *Pseudo-nitzschia* was amplified and sequenced. The secondary structure of ITS2 transcript of the Sarawakian strains and their related taxa was predicted and finally used to infer the phylogenetic framework of the genus *Pseudo-nitzschia*.

2.0 LITERATURE REVIEW

2.1 Harmful Algal Blooms

Harmful Algal Blooms (HABs) is a natural phenomenon due to drastic increase in the density of some microalgae species that has caused deleterious effect to the ecosystems including the fauna and flora. Most of the HABs events were resulted by the blooms of dinoflagellate species with few exceptions. For instance, amnesic shellfish poisoning (ASP) is caused by toxic diatom *Pseudo-nitzschia* spp.

Pseudo-nitzschia spp was widely grown through sea waters of both warm and cold climates. During the spring and autumn, the heavy rainfall and nutrients runoff most likely increased the numbers of phytoplankton blooms (Bates et al., 1989). Blooms were formed by the rapid growth of phytoplankton. Phytoplankton accumulates in warmer surface waters because of the low wind and the water current. The production of domoic acid (DA) by different species of *Pseudo-nitzschia* is affected by environmental factors such as light and temperature. The warmer seawaters temperature (14 – 17^{o}C) was needed by some *Pseudo-nitzschia* species to produce high concentration of domoic acid (Walz et al., 1994). Lower temperature was needed by the some *Pseudo-nitzschia* species (*P. seriata*) to produce high concentration of domoic acid (Lundholm, 1994).

HABs cases related to paralytic shellfish poisoning (PSP) have been reported in Malaysia. These events were reported at Kota Kinabalu, Sabah, Sebatu, Melacca, and Tumpat, Kelantan. There were many types of shellfish poisoning caused by the HABs. Among the cases, there was no ASP cases had yet been reported in Malaysia. However studies carried out in the Malaysian waters showed that *Pseudo-nitzschia* spp. is widely distributed in our waters (Su et al., 2009).

2.2 Amnesic Shellfish Poisoning (ASP)

Outbreak of Amnesic shellfish poisoning had been reported for the first time in Canada at 1987. Approximately 150 reported cases with 19 hospitalized and 4 deaths after consumption of contaminated mussels (Perl et al., 1990). Other 107 individuals just have common gastrointestinal symptoms with vomiting (76%), abdominal cramps (50%) and diarrhea (42%) and the most common neurological symptoms were severe headache (43%) and loss of short-term memory (25%) (Perl et al., 1990). Besides, there were 21 individuals with gastrointestinal and/or mild neurological symptoms after consumption of razor clams in Washington State (USA) in 1991.

2.2.1 The neurotoxin, Domoic acid

Domoic acid is one of the amino acid belongs to the kainoid class of compounds (Wright et al., 1990). Ten types of domoic acid isomer had been discovered (Maeda et al., 1986; Zaman et al., 1997). For example, isodomoic acid A to H. Domoic acid is water soluble and do not degrade under ambient temperature. However, domoic acid could be decomposed under acid condition (Quilliam et al., 1989).

The domoic acid had been proven to accumulate in a variety of shellfish. Cockles (*Cerastoderma edule*), crabs (*Cancer magister*), furrow shell (*Scrobicularia plana*), mussels (*Mytilus edulis*), razor clams (*Siliqua patula*) and scallops (*Pecten maximus*) had been reported to contain domoic acid (Wekell et al., 1994; Vale & Sampayo, 2001).

According to Perl et al. (1990), ingestion of ~60 mg DA/person would cause gastrointestinal symptom and 270 mg DA/ person would induce neurological effect. There are seven species of the *Pseudo-nitzschia* spp. had been reported to produce DA in either cultured or natural populations. They are *P. austrulis, P. delicatissima* (Cleve)

Heiden, *P. multiseries* (Hasle) Hasle, *P. pungens* (Grunow) Hasle, *P. pseudodelicatissima* (Hasle) Hasle, *P. seriata* (Cleve*) Peragallo, and P. turgidula* (Hustedt) Hasle.

Pseudo-nitzschia australis was first found at San Matías, Argentina (Frenguelli, 1939). It produces domoic acid and isodomoic acid (Frenguelli, 1939). *P. delicatissima* was first found in the North Atlantic, Spitzbergen and Sweden (Heiden, 1982). *P. multiseries* had been found in Drøbak, Oslofjord, Norway (Hasle, 1965). It also widely distributed at Korea, Japan and New Zealand. *P. pungens* was first discovered at Yeddo Bay, Japan (Hasle, 1993). It was usually non-toxic. However, toxic clones had been reported from New Zealand and the West Coast of the U.S.A. P. seriata (H. Peragallo, 1897-1908) had been first found at Tindingen, Greenland. *P. turgidula* (Hasle, 1993) had been first discovered at two localities in the South Atlantic. A strain from New Zealand was tested positive for DA.

2.3 Characterizations of the diatom *Pseudo-nitzschia* spp.

2.3.1 Morphological characterization

Diatom is commonly classified by the morphological characterization based on the morphometric measurement. Some parameters had been used in this measurement. They are valvar length and width, fibula, stria and poroid densities, number of sector within paroids (Hasle, 1965). The combination of the morphological characteristic and genetic characteristic have revealed several cryptic and pseudo-cryptic species in both centric (Sarno et al., 2005) and pennate diatoms (e.g. Lundholm et al., 2003; Amato et al., 2007; Vanormelimgen et al., 2008). Cryptic species was the species that had been reported to have morphologically identical and different molecular structure. The pseudo-cryptic is the species that showed genetic differences and also minor ultrastructural differences (Mann & Evans, 2007). This method had been used widely for *Pseudo-nitzschia* spp.

characterization, however, morphological characterization have been only used in certain *Pseudo-nitzschia* spp. This is because some of the *Pseudo-nitzschia* spp. had the same morphological structure to hide themselves to be discovered by scientists.

2.3.2 Sexual compatibility

Sexual reproduction of diatom had been used to develop the sexual compatibility characterization. For most of the *Pseudo-nitzschia* spp., sexual reproductions was the only way out of this miniaturization trap, and involve meiosis, gamete fusion, and the formation of a specialized zygote called auxospore (Chepurnov et al., 2004). Almost all *Pseudo-nitzschia* spp. had been discovered to have heterothallic mating system, with one exception, *Nitzschia subcurvata* (Fryxell et al., 1991), in which auxospore were observed in a monoclonal culture. Sexual reproduction could not be the most suitable way to characterize the *Pseudo-nitzschia* spp. because almost all *Pseudo-nitzschia* spp. have heterothallic mating system.

2.3.3 Molecular characterization

2.3.3.1 Nuclear encoded small subunit and large subunit rRNA genes

SSU rRNA gene had been used to infer phylogenetic relationships of *Pseudo-nitzschia* spp. (Van der Auwera & De Wachter, 1998; Ludwig & Klenk, 2001). Diatom gene study research involved phylogenetic reconstruction to study the evolution of the major classes (Medlin & Kaczmarska, 2004), to assess the monophyly of diatom orders or genera (Beszteri et al. 2001) and to investigate the presence of cryptic species (Sarno et al., 2005). The rDNA have been used to solve the problem of monophyletic origin of the

raphid diatom (Philippe et al., 1994). SSU rDNA was used to indicate that the Bacillariaceae is monophyletic (Medlin et al., 2000).

For the LSU rRNA gene, it has more variable regions when compared to the SSU rRNA gene (Van der Auwera & De Wachter, 1998). The LSU rRNA was used to solve the phylogenetic closely related species, but it might cause reconstruction of deep phylogenetic problem because of saturation effects. According to Nina Lundholm et al. (2002), they showed that LSU rDNA was more suitable and efficient to solve the monophyletic problem compared to the SSU rDNA. This is because LSU rDNA contains highly variable regions and useful marker to elucidate the phylogenetic relationship within Bacillariophyceae (*Pseudo-nitzschia*).

2.3.3.2 Internal Transcribed Spacer (ITS) Region

The internal transcribed spacer region (ITS) was referred to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNAs) on a common precursor transcript (Figure 2.1). There are two type of ITSs, the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2).

ITS1 and ITS2 are transcribed regions but there are not parts of the mature ribosomes. There have complex secondary structure. They play a crucial role in the construction of ribosomes (Tschochner & Hurt, 2003).

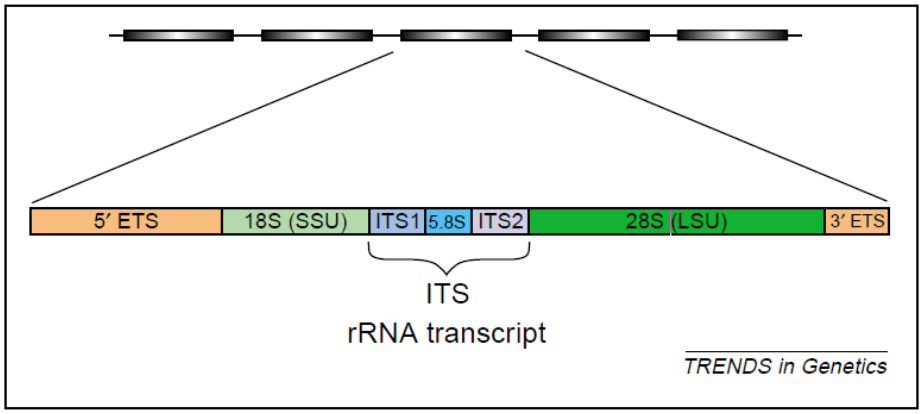

Figure 2.1: Single repeat of eukaryotic ribosomal RNA gene precursor with the small subunit (18S), 5.8S, large subunit (28S), the first internal transcript spacer (ITS1) and the second internal transcript spacer (ITS2) as well as the external transcript spacer (adopted from Coleman, 2003).

For molecular studies of the *Pseudo-nitzschia* spp., ITS2 region had been used to analyze and showed distinct eukaryotic hallmarks (Figure 2.2). There are four main helices, of which helix III is the longest, the presence of a characteristic motif at the apex of helix III, and a pyrimidine mismatch in helix II (Schultz et al., 2005). There are very variable region compare to the LSU and SSU. ITS2 region is good information to infer the phylogenetic work.

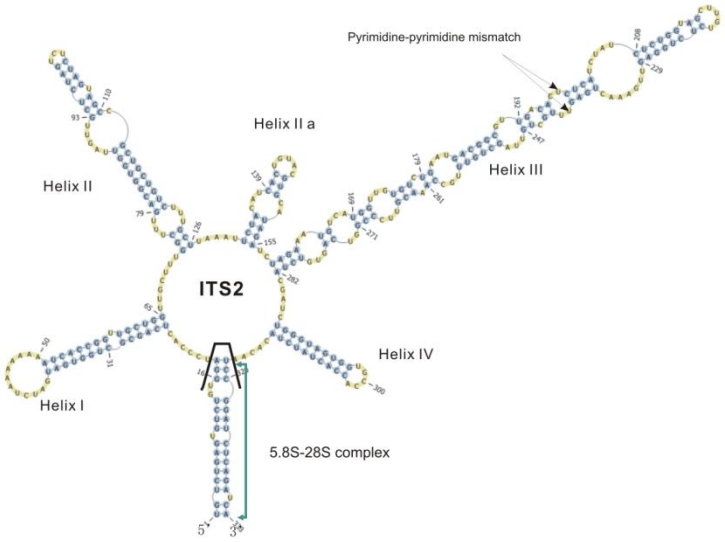

Figure 2.2: Secondary structure of the second internal transcribed spacer (ITS2) transcript of *Pseudo-nitschia mannii*.

There are many bioinformatics programs with different algorithm used to predict the secondary structure of RNA. The most common algorithm used to predict the ITS2 secondary structure is the free energy minimization (Mathew and Turner, 2006). Although the free energy minimization was very convenience but it has limitations in sub-optimal structure predictions since some of naturally occurred RNAs are usually free energy sub-optimum. The limitations of free energy minimization included approximations (Mathew and Turner, 2006), the algorithm also folded the sequences into more than one structure and the RNA sequences adopt secondary structure that were partially determined by folding algorithm (Schultes et al., 2000).

Current RNA secondary structure predictions have been improved and statistical mechanisms and algorithm that allow the prediction of pseudoknots as well as structure prediction based on covariance model and homologous modeling (Mathew and Turner, 2006).

3.0 MATERIALS AND METHODS

3.1 Algae culture

3.1.1 Medium preparation

SWII medium (Table 3.1; Iwasaki, 1961) was used for culturing in this study. 1 mM of silicate was added for *Pseudo-nitzschia* culturing. Twenty-five mililiter of SWII medium was transferred into test tubes and autoclaved immediately after the preparation. The medium was left for 2 days before being used in culturing.

Table 3.1: SWII Medium (Iwasaki, 1961)

Component [concentration]		Volume	Final concentration
KNO_3	[7.2×10^{-3} mol/L]	1.0 mL	[7.2×10^{-4} mol/L]
KH_2PO_4	[3.31×10^{-4} mol/L]	1.0 mL	[3.31×10^{-5} mol/L]
Na_2-glycero.PO_4	[3.33×10^{-4} mol/L]	1.0 mL	[3.33×10^{-5} mol/L]
Vitamin mix		1.0 mL	
• 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 mL	[1.19×10^{-6} mol/L]
Tris-HCl (pH 7.8)		1.0 mL	[4.13×10^{-3} mol/L]

Make final volume up to 1.0 L with ddH_2O.

3.1.2 Culturing

Algal culturing was carried out aseptically in the laminar flow hood. The cover and mouth of the test tube were flamed before and after inoculation. Approximately 5 mL of stock culture was inoculated into a new tube with SWII medium. Cultures were then kept in a temperature-light controlled growth chamber (ShelLab, USA) and maintained at 26 ± 0.5^{o} C under 14:10 hour light:dark photo period. Transferring of batch cultures into new medium was conducted every week (7 day-intervals) to maintain a good condition cultures. The cultures used in this study are clonal but non-axenic. The cultures were obtained from UNIMAS harmful algae culture collection in the Laboratory of Ecotoxicology.

3.2 Genomic DNA extraction

Pseudo-nitzschia clonal cultures at late exponential phase were harvested for genomic DNA extraction. One test tube of culture (approximate 25 mL) was transferred into a 50 mL centrifuge tube for cell harvesting. The samples were centrifuged at 10,000 rpm for 10 minutes. The excess medium was discarded. The cell pellet was resuspended in a small amount of medium and transferred to a 1.5 mL microfuge tube. The sample was centrifuged again and supernatant was discarded.

A total of 700µL of 2× Cetyl-trimetyl-ammonium-bromide (CTAB) buffer was added to the cell pellet. Subsequently 5 µL of Proteinase K (2mg/ml) was added. The sample was mixed vigorously by vortexing. The sample was then incubated in water bath at 60^{o} C for 45 minutes to 1 hour.

After incubation, 700µL of chloroform:isoamyl alcohol (24:1) was added to the mixture. The sample was mixed well by inverting the centrifuge tube. The sample was

centrifuged at 10,000 rpm for 10 minutes. The sample was fractioned into two layers. The upper aqueous phase was transferred into a new centrifuge tube.

For DNA precipitation, approximately 500µL of absolute ethanol (EtOH) and 25µL sodium acetate 3M (NaAOc) were added. The centrifuge tube was inverted for mixing the solution. The sample was kept in -20^{o} C for 3 hours.

After 3 hour, the sample was centrifuged again at 13,000 rpm for 10 minutes. The excess ethanol was discarded. This followed by adding 500 µL of 70% ethanol to the sample pellet and mixed by taping the tip of the tube. The sample was then centrifuged at 13,000 rpm for 10 minutes. The excess ethanol was poured out and left the DNA pellet. The DNA sample was dried at room temperature for a few minutes.

The DNA pellet was dissolved in 30µL of TE (10mM Tris-HCl, PH7.4; 1mM EDTA, pH 8.0) buffer. The sample was let stand at 4^{o}C overnight. The DNA sample was then stored at -20^{o} C freezer for long-term storage (< 6 months).

3.3 DNA quantification and qualification

Spectrophotometric method was used to quantify the DNA concentration. A total of 3,996 µL of ddH_2O and 4µL of genomic DNA were added to the cuvette. The samples were mix thoroughly and measured at the wavelength of 260 nm and 280 nm using the Libra S12 spectrophotometer (Biochrom, United Kingdom). DNA concentration was determined using the equation as below.

$$[DNA] = OD_{260} \times Dfactor \times 50\ \mu g\ mL^{-1}$$

where OD_{260} is the optical density at wavelength of 260 nm, Dfactor is the dilution factor (10^{-3} in this study), and 1 OD_{260} of double stranded DNA is equivalent to 50 µg mL^{-1}.

The quality of genomic DNA was determined by the ratio between OD at 260 nm and 280 nm. The ratio of 1.7 was considered as high purity DNA.

3.4 ITS region of nuclear encoded ribosomal RNA gene amplification

Ribosomal gene amplification was carried out by polymerase chain reaction (PCR). Amplification of ITS region of *Pseudo-nitzschia* species was conducted by using the primers ITS1 (5' TCCGTAGGTGAACCTGCG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Vivantis, Selangor) developed by White et al. (1990). The PCR mixtures were prepared as follow (Table 3.2). The Fermentas Dream-Taq *Taq* polymerase (Fermentas, USA) was used in this study.

Table 3.2: PCR Reagent (Fermentas)

Reagents	Stock concentration	Final Concentration	Volume (µL)
Buffer + $MgCl_2$	10× (20 mM $MgCl_2$)	1× (2mM $MgCl_2$)	2.5
ITS1	25 µM	1 µM	1.0
ITS4	25 µM	1 µM	1.0
dNTPs	25 mM	0.2 mM	0.2
Taq	5U μL^{-1}	1U	0.2
DNA Template		1-100 ng	2.0
dH_2O			18.1
Total volume			25.0

PCR was conducted using the Eppendorf gradient thermocycler (Eppendorf, Germany). The thermal cycling steps used in this study were as below (Table 3.3). The total cycle in the PCR reaction was 35 cycles.