Faculty of Resource Science and Technology

Molecular characterization of the diatom, *Pseudo-nitzschia* (Bacillariophyceae) using the mitochondrial genetic marker, cytochrome oxidase I (COI)

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This project is submitted in partial fulfillment of the requirements for the degree of
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DECLARATION

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

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LIST OF ABBREVIATIONS

ASP	Amnesic shellfish poisoning
BI	Bayersian analysis
BLAST	Basic local alignment tool
CI	Consistency index
COI	Cytochrome oxidase I
CTAB	Cetyl-trimetyl-ammonium-bromide
DA	Domoic acid
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EtBr	Ethium bromide
EtOH	Ethanol
HABs	Harmful algal blooms
HI	Homoplasy index
LM	Light microscopy
LSU	Large-subunit
ML	Maximum likelihood
MP	Maximum parsimony
NCBI	National center of biotechnology information
PAUP*	Phylogenetic analysis using parsimony*
PCR	Polymerase chain reaction
RC	Rescaled consistency index
rDNA	Ribosomal deoxyribonucleic acid
RI	Retention index
SEM	Scanning electron microscopy
SSU	Small-subunit
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy

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ABSTRACT

Pseudo-nitzschia is a marine and planktonic chain-forming diatom. Some *Pseudo-nitzschia* species produce biotoxin, domoic acid (DA) which causes Amnesic Shellfish Poisoning (ASP) in humans and marine mammals. Species of *Pseudo-nitzschia* are difficult to distinguish under light microscope (LM). In this study, a total of 22 strains of *Pseudo-nitzschia* from Malaysian waters were identified based on morphological characteristics observed under LM and transmission electron microscope (TEM). A total of 17 stains were successfully identified; they are *P. circumpora*, *P. cuspidata* and *P. pungens*. A pair of genus-specific primer targeting the mitochondrial cytochrome oxidase I (COI) gene was developed *in silico* to infer the molecular phylogeny of *Pseudo-nitzschia* species. The primer specificity was tested using genomic DNA from clonal cultures. The designed primer was successfully amplified with a fragment length of 450 bp. A total of 12 strains of *Pseudo-nitzschia* species were successfully amplified. LSU rRNA gene of the cultures were also amplified to compare with the COI dataset. Genetic divergences of COI dataset and LSU rRNA dataset among the *Pseudo-nitzschia* taxa were ranged from 0 to 20.5 % and from 0 to 5 % respectively. Phylogenetic reconstruction revealed *Pseudo-nitzschia* taxa as a monophyletic group. However, the results showed incongruence in both COI and LSU phylogenies. As conclusion, the cultures and the species identity obtained in this study provide useful information on *Pseudo-nitzschia* study, and added to the database of harmful algal blooms research in the country.

Key words: *Pseudo-nitzschia*, Amnesic Shellfish Poisoning (ASP), cytochrome oxidase I (COI), *in silico*, LSU rRNA

ABSTRAK

Pseudo-nitzschia merupakan salah satu diatom marin, planktonik dan berkoloni rantaian. Sesetengah spesies Pseudo-nitzschia berkemampuan menghasilkan biotoksin, iaitu asid domoik (DA) yang mengakibatkan keracunan kerang-kerangan amnesik (ASP) pada manusia dan mamalia laut. Spesies Pseudo-nitzschia sukar dicam di bawah mikroskop cahaya (LM). Dalam kajian ini, sebanyak 22 jenis Pseudo-nitzschia dari perairan Malaysia telah dikenalpasti berdasarkan ciri-ciri morfologi yang diperhatikan di bawah mikrskop cahaya (LM) dan mikroskop electron transmisi (TEM). Sebanyak 17 klon telah dikenalpasti, mereka terdiri daripada P. circumpora, P. cuspidata dan P. pungens. Sepasang penjujuk mensasarkan gen mitokondria sitokrom oksidase I (COI) telah direkabentuk secara in silico untuk menyimpulkan filogeni molekul spesies Pseudo-nitzschia. Spesifisiti penjujuk tersebut telah diuji dengan menggunakan DNA genomik dari kultur-kultur klon. Penjujuk tersebut telah berjaya menjujuk satu produk gen dengan panjangnya 450 bp. Sejumlah 12 klon spesies Pseudo-nitzschia telah berjaya dijujuk. Gen LSU rRNA juga dijujukkan untuk perbandingan dengan set data COI. Perbezaan genetik set data COI antara jenis Pseudo-nitzschia adalah 0 ke 20.5 % manakala set data LSU rRNA adalah 0 ke 5 %. Pembinaan semula filogenik mendedahkan taksa Pseudo-nitzschia sebagai satu kumpulan monofiletik. Walau bagaimanapun, keputusan kajian ini menunjukkan topologi yang berbeza dalam kedua-dua rangka filogeni COI dan LSU. Secara kesimpulan, kultur-kultur dan identiti spesies yang diperolehi dalam kajian ini telah menyediakan maklumat yang berguna di kajian Pseudo-nitzschia, dan menambahkan pangkalan data penyelidikan ledakan alga berbahaya di negara ini.

Kata kunci: Pseudo-nitzschia, keracunan kerang-kerangan amnesik (ASP), sitokrom olsidase I (COI), in silico, LSU rRNA

1.0 INTRODUCTION

Diatoms are ecologically widespread and the most species rich algae (Kaczmarska *et al.*, 2008). *Pseudo-nitzschia* was first identified by H. Peragallo (Lelong *et al.*, 2012). *Pseudo-nitzschia* species is one of the common algae that causing harmful algal blooms (HABs) in Malaysia. In the year of 1965, 18 species and two subspecies were identified in the group of *Pseudo-nitzschia*, such as *P. actydrophila, P. barkleyi, P. cuspidata, P. delicatula, P. fradulenta, P. granii, P. heimii, P. inflatula, P. lineola, P. prolongatoides, P. pseudoseriata, P. pungens, P. pungens* f. *multiseries, P. seriata, P. seriata* f. *obtusa, P. subcurvata, P. subfraudulenta, P. subpacifica, P. turgidula* and *P. turgiduloides* (Hasle, 1964; 1965).

Priisholm, Moestrup and Lundholm (2002) claimed that *Pseudo-nitzschia* is a genus of chain-forming planktonic diatom that can produce neurotoxin domoic acid (DA) such as *P. australis, P. fraudulenta, P. multiseries, P. multistriata, P. pseudodelicatissima, P. pungens, P. seriata* and others. These species that produce toxins are mostly harmful to marine mammals and humans (Kaczmarska *et al.*, 2008). Sullivan (1993) as cited in Falconer (1993) claimed that amnesic shellfish poisoning is a human illness that caused by the diatom, *Pseudo-nitzschia* that produce DA in shellfish.

Morphological observation and ultrastructure investigations are the main approach in identification of *Pseudo-nitzschia* species. According to Lelong *et al*. (2012), molecular studies such as sequence analyses of the nuclear-encoded large-subunit (LSU) ribosomal RNA gene (rDNA), the internal transcribed spacer (ITS1 or ITS2) regions and others have been used to develop an automated ribosomal intergenic spacer analysis approach to identify *Pseudo-nitzschia* species. Recently, there is much information about *Pseudo-nitzschia* species identification, morphological, toxicity and others have been study but

there are still a lot of others *Pseudo-nitzschia* species that present in the seawater in the world. However, studies about molecular characteristics of *Pseudo-nitzschia* by using mitochondrial gene markers were very limited.

The main objective of this project was to study the molecular characterization of *Pseudo-nitzschia* species from Malaysia by using the mitochondrial genetic marker, cytochrome oxidase I (COI) gene in order to support the morphological identification and phylogenetic relationship of *Pseudo-nitzschia*, which is useful for solving phylogenetic resolution. The information of the unambiguous sequence alignment of COI was used to reconstruct a reliable phylogenetic framework to infer the phylogenetic lineage of the species in the genus. Besides that, there were some specific objectives as below:

1. To identify the species of *Pseudo-nitzschia* from Malaysian waters based on morphological observation;
2. To obtain the nucleotide sequences of COI gene from *Pseudo-nitzschia* spp.;
3. To evaluate COI gene as a suitable molecular marker of *Pseudo-nitzschia* spp.

2.0 LITERATURE REVIEW

2.1 Harmful algal blooms (HABs)

In Malaysia, there was a case which HABs and shellfish toxicity happened in Brunei Bay on the west coast of Sabah. This was the first time that marine dinoflagellate *Pyrodinium bahamense* var. *compressum* bloomed. Besides that, paralytic shellfish poisoning (PSP) is the most popular seafood inotoxication due to algal toxins such as viz. *P. bahamense* var. *compressum* in Sabah, *Alexandrium tamiyavanichii* in Sebatu, Malacca and *A. minutum* in Tumpat, Kelantan (Fukuyo *et al.*, 2011).

Harmful algae are phytoplankton that serve as energy provider in the marine ecosystem but multiplication of algal blooms until it reach high concentrations of phytoplankton cells in the coastal water will cause harmful algae blooms, also known as 'red tides'. Macronutrients such as silicon, phosphorus and nitrogen and micronutrients such as trace metals in seawater are leading to the formation of HABs (Maso and Garcés, 2006).

The development of the ecosystem in the coastal waters can be affect by HABs and these HABs will give negative impacts toward humans especially for those fishermen that will be relied on the goods that in the coastal and marine ecosystems. There are several types of shellfish poisoning that cause by the HABs which impact the human health, such as paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP) and ciguatera fish poisoning (CFP) (Zingone and Enevoldsen, 2000). The diatoms of *Pseudo-nitzschia* will produce the neurotoxins that responsible to ASP and those who accidentally ate the shellfish which contaminated with DA especially humans will cause human intoxication.

2.2 Amnesic shellfish poisoning (ASP)

According to Sullivan (1993) as cited in Falconer (1993, p.43), ASP is one type of marine intoxication that has been reported in the year of 1987 at off Prince Edward Island, Canada. That time, there were about 156 victims ate blue mussels which contaminated with the neurotoxin domoic acid (DA), $C_{15}H_{21}NO_6$ (Figure 2.1) that produced *P. pungens*. Apart from this, there were three persons died and 105 cases of acute human intoxication after contaminated with DA. People who consumed high concentration of ASP will have the same symptoms as Alzheimer's disease which is loss of short-term memory (Lim *et al.*, 2010). Besides that, symptoms of ASP such as diarrhea, nausea, vomiting, headache and other also can be from those who involve in ASP. In Malaysia, there is no reported case of ASP. There were only little study of ASP but some of the species have been established for identification and toxicity screening in order to increase the molecular data of *Pseudo-nitzschia* species.

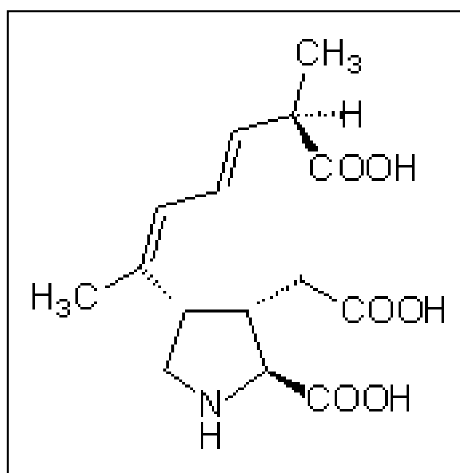

Figure 2.1: Structural formula of domoic acid (DA) (adopted from Lim *et al.*, 2010).

2.3 The genus *Pseudo-nitzschia* species

There were 37 species known in the genus *Pseudo-nitzschia* (Lelong *et al*., 2012). Type species of the genus called *P. seriata* has been renamed from *Nitzschia seriata* and 14 of the species have been transferred from *Nitzschia* to *Pseudo-nitzschia*. The genus *Pseudo-nitzschia* is divided into two subgroups, *seriata*- and *delicatissima*- group. The width of valve of *Pseudo-nitzschia* in the *seriata*-group is more than three micron than the width of valve in the *delicatissima*- group (less than three micron) (Hasle and Syvertsen, 1997).

According to Graneli and Turner (2007), they mentioned that *P. brasiliana*, *P. subfraudulenta* and *P. subpacifica* can be found in warmer waters whereas *P. pungens* is less or more cosmopolitan. Apart from this, Graneli and Turner (2007) also claimed that *P. americana, P. australis, P. calliantha, P. delicatissima, P. fraudulenta and P. multiseries* are cosmopolitan. According to Parsons *et al.* (1999), the genus of *Pseudo-nitzschia* affected human health cause of people eating mussels that contaminated with DA previously. It was viewed as a universal and innoxious member of the phytoplankton where it gave large number of carbon biomass (Trainer *et al*., 2012).

Trainer *et al*. (2012) stated that cryptic species are same in the form of morphology but they have different genetic among them. Apart from this, pseudo-cryptic species have less different in genetic and morphology. In Malaysia, according to Teng *et al.* (Submitted), there were 22 species of *Pseudo-nitzschia* have been found in Malaysia waters such as *P. Americana, P. brasiliana, P. caciantha, P. calliantha, P. circumpora, P. cuspidata, P. decipien, P. delicatissima/ P. arenysensis, P. dolorosa, P. inflatula, P. linea, P. lineola, P. mannii, P. micropora, P. multistriata, P. pseudodelicatissima, P. pungens, P. sinica, P. subfraudulenta* and *P. turgidula.*

Pseudo-nitzschia is a paraphyletic genus that has been proves by LSU rRNA sequence analysis. *Pseudo-nitzschia* species are pennate diatoms with a longitudinal symmetry and they are able to form chains except the *P. americana* which exists as single cells (Lelong *et al.*, 2012). Their cells in a chain overlap their neighbor cells at the tips (Lim *et al.*, 2012). All species will have the same general morphology such frustules that made up of two valves, which is a cell wall that made of silicic acid, $Si(OH)_4$ (Lelong *et al.*, 2012).

Sexual reproduction of *Pseudo-nitzschia* involves meiosis, gamete fusion and the formation of auxospore (specialized zygote). Almost all have a heterothallic mating system except *P. subcurvata* that has been reported (Amato *et al.*, 2007). According to Trainer *et al*. (2012), the size of the cells in clonal cultures of *Pseudo-nitzschia* die or decreased over time if they do not undergo the process of auxosporulation or sexual reproduction. Besides that, they are hard to produce DA except for *P. multiseries*, where their offspring can be toxic.

Based on the tool of morphology identification for *Pseudo-nitzschia* species, it is more viable by using scanning electron microscopy (SEM) or transmission electron microscopy (TEM) better than using light microscopy (LM) but length, width and cell shape of *Pseudo-nitzschia* species can be determined through LM (Lelong *et al.*, 2012). Features such as absence or presence of large central interspace in the cell, valve shape, number of striae and poroids can be used to differentiate the species in the genus.

2.4 Molecular phylogeny of the genus *Pseudo-nitzschia*

Phylogenetic analysis of the genus *Pseudo-nitzschia* are commonly performed based on partial large subunit (LSU) rDNA and the internal transcribed spacer (ITS) regions, but less on COI gene.

2.4.1 Nuclear-encoded genes, ribosomal DNA (rDNA)

According to Alverson (2008), the nuclear rDNA was the most widely sequenced markers due to the highly conserved regions and the hypervariable regions give better phylogenetically informative characters. According to (Lundholm *et al*., 2002), the nuclear-encoded large subunit (LSU) rDNA indicated stronger phylogenetic signal than the small subunit (SSU) rDNA. LSU rRNA is more helpful on resolve species- and population-level relationships while SSU rDNA is more on reconstructing higher level relationships across the entire phylogeny of diatoms (Alverson, 2008). Apart from this, in the study of Orsini *et al.* (2002), hypervariable domains (D1-D3) LSU rRNA showed good results on to the phylogenetic relationships among several *Pseudo-nitzschia* species.

2.4.2 Mitochondrial cytochrome oxidase I (COI) gene

Cytochrome oxidase I (COI) gene is a mitochondrial protein that can be found in the mitochondrial membrane and its function as an enzyme in the electron transport chain (Struder-Kypke and Lynn, 2009). According to Alverson (2008), the mitochondrial genome in diatoms acts as a tools for phylogenetic studies because of their small size and low polymorphism of mitochondrial genes. Besides that, only COI gene and cytochrome b have been investigated for phylogenetic utility among the protein-coding genes in diatom

mitochondrial genomes. As the result, it shown that multiple levels of phylogentic relationship can be solved by these mtDNA within diatoms.

Apart from this, Wan *et al.* (2004) claimed that mitochondrial DNA (mtDNA) in phylogeny is appropriate for resolving taxonomic uncertainties in conservation genetics but mtDNA has a relatively high mutation fixation rate due to its DNA is histone-free and it has limitation on the ability in repair. Mitochondrial protein-coding genes evolve faster and more powerful marker for inferring the evolution history compares to the 12S rDNAs and 16s rDNAs. Evans, Wortley and Mann (2007) concluded that COI gene sequences were more divergent than chloroplast rbcL sequences and it is easier to align if compare to ITS rDNA sequences.

According to Zhang and Hewitt (2003), there are some important limitations of mtDNA which are the mtDNA represents only a single locus and the effective population size of mtDNA is only a fourth compare to nuclear autosomal sequences. Until now, there is no research on molecular characterization of *Pseudo-nitzschia* species by using COI gene.

3.0 MATERIALS AND METHODS

3.1 Sample collection

Plankton samples were collected using a 20 μm mesh-size plankton net at the coastal waters of Santubong, Semariang Batu and Muara Tebas during high tide. The plankton net was towed vertically within the first 5 m about four to five times. The samples were kept in 500 mL bottles and brought back to the laboratory for cell isolation (Lim *et al.*, 2012).

3.2 Cell isolation and algal culture

The cultures were observed under a light microscopy for genus confirmation. The species were isolated from the sampling water and established into clonal culture. First, single cell isolation were picked up from the sampling water by micro-pipette isolation technique under an Olympus IX71 inverted light microscope (Olympus, USA) within two days after sampling to prevent the cells die off. Then, the single cell was transferred onto a glass slide that contains ambient filter-sterile seawater (filter from the sampling seawater). The cell was rinsed with the ambient filter-sterile seawater a few times to remove detritus and epiphytes. Approximately 100 μL of ambient filter-sterile seawater was added into each well of the 96 multi-well plates. After that, each of the single rinse cells was transferred into each well.

After cell isolation, the multi-well plate was kept into the incubator. The species were observed once in every two days. Few drops of f/2 medium (Guillard and Ryther, 1962; Guillard, 1975) were added into each well every two or three days. The cell was removed into a 24 multi-well plates that contain f/2 medium by using a micropipette (100 - 200 μL) to let the cells divide again. Cells were transferred into sterile 25 mL test tubes

that contained f/2 medium when it reached more than 100 cells. Cultures were grown and maintained in the f/2 medium with pH of 7.9, 30 PSU and under 12:12 hour light: dark photoperiods with light intensity of 200 μmol photons $m^{-2}s^{-1}$ at the temperature of 25±1 ℃ (SHELAB, USA).

Clonal cultures of *Pseudo-nitzschia* species in this study was obtained from the UNIMAS Harmful Algae Collection. A total of 22 clonal cultures of *Pseudo-nitzschia* spp. were maintained in this study.

3.2.1 Medium preparation

In this study, f/2 medium was used (Guillard and Ryther, 1962; Guillard, 1975). The medium was prepared by using the seawater as the medium base. The salinity of the medium was adjusted to approximately 30 PSU by diluting the natural seawater with distilled water. One litre of filtered seawater (0.2 μm) that have been autoclaved were mixed with 1 mL of silicate stock solution ($Na_2SiO_3.9H_2O$), 1 mL of sodium hydrogen phosphate ($NaH_2PO_4.H_2O$), 1 mL of sodium nitrate ($NaNO_3$), 1 mL of trace metal solution (Appendix A) and 500 μL of vitamin solution (Appendix A). The pH of the seawater was adjusted to 7.8-7.9 (Guillard, 1975). The medium was left overnight before inoculation.

3.2.2 Sub-culturing

Process of subculture was done in laminar flow hood with aseptic techniques every week or when the cells concentration of clonal cultures is high in order to maintain the cultures. All cultures were maintained in f/2 medium with the enrichment of silicate. A clean test tube was used which soaked in 10% HCl overnight and rinsed with tap water and distilled

water. First, the distilled water that inside the test tube was poured out which have been autoclave before. Then, approximately 45 % of the test tube was filled with f/2 medium. Lastly, 2 mL of cultures were transfer into the medium.

3.3 Morphological observation by TEM

Morphology of *Pseudo-nitzschia* species were observed under electron microscopy. Prior to TEM observations, samples were undergone the process of acid wash (Bargu *et al.*, 2002). One mililitre of plankton samples were transferred into a 1.5 mL microfuge tube and centrifuged for 10 min at 10 000 rpm. Then, the pellet of cells were treated with potassium permanganate ($KMnO_4$) and left for 15 min. Hydrochloric acid (HCl) was added into the sample and the mixture will be left for 30 min in order to remove organic matters. 10% oxalic acid was added in order to get clear solution. The sample was then rinsed with distilled water until the cell suspension become less acidic. One drop of acid wash clean cells was transferred onto a Fomvar-coated copper grid and air dried. Do not add too much of samples on the copper grid to prevent cell overlapping in the copper grid. The samples were observed under JEOL JEM-1230 TEM (JEOL, Japan). Detail morphological features such as length, width, number of striae, number of fibulae, number of poroids, shape of sector in poroid, number of valvopula and others were observed through electron microscopes.

3.4 Molecular analysis

3.4.1 DNA isolation

Genomic DNA of *Pseudo-nitzschia* clonal cultures was harvested during late exponential growth phase (around 4-5 days after subculture) or during the high cells concentration of the clonal cultures. First, the dense clonal culture was pipette into microfuge tube and centrifuged at 6000 rpm for 15 min. The medium was pipette and finally the pellet was left in the tube. 700 μL of 2×Cetyl-trimetyl-ammonium-bromide (CTAB) buffer that contains 20 mM ethylenediaminetetraacetic acid (EDTA), 50mM CTAB, 10mM Tris-Base, 14 mM NaCl and 0.2 M 2-β-mercaptoethanol was added. A total of 5 μL of Proteinase K (2 mg/mL) were added into the mixture. The mixtures were mixed and incubated in water bath at 65 ℃ for 45 to 60 min. After incubation, 700 μL of chloroform: isoamyl alcohol was added quickly. The mixtures was vortex and centrifuged at 10 000 rpm for 10 min. The upper layer in the tube was transferred into a new centrifuge tube and mixed by inversion. For DNA precipitation, 500 μL of absolute ethanol, EtOH and 25 μL of sodium acetate (NaAOc) were added. The centrifuge tube was inverted in order to mix the solution. The sample was then kept in -20 ℃ freezer for three hours. The mixture was centrifuged at 13 000 rpm for 10 min. Excess ethanol was poured out. 500 μL of 70 % of cold EtOH was added and the mixtures were mixed by taping the tip of the tube. The mixtues was then centrifuged again at 13 000 rpm for 10 min. EtOH was removed and the excess EtOH which located at the side of the inner tube was removed by an autoclave cotton bud. DNA sample was air dried at room temperature for 30-60 min and re-suspended with 30 μL of ddH_2O or TE (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0) buffer. The genomic DNA was stored at -20 ℃ for further analysis and long term storage.