RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING OF MARINE HARMFUL ALGAE, GENUS *PSEUDO-NITZSCHIA*

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THIS THESIS IS PRESENTED TO FULFILL THE REQUIREMENT TO OBTAIN A BACHELOR OF BIOTECHNOLOGY DEGREE WITH HONOURS

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DECLARATION

I hereby declare that this is my original work except for the quotations that I have clearly stated in the sources.

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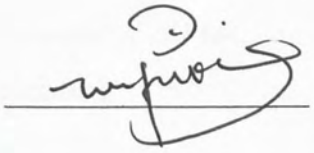

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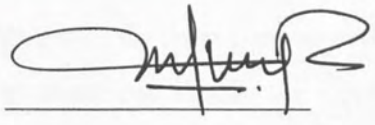

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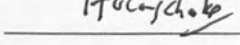

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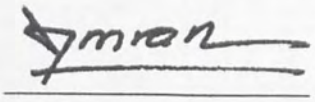

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ABSTRACT

In this study, the Random Amplified Polymorphic DNA (RAPD) technique was used to distinguish three algae species of the genus *Pseudo-nitzschia*; PNA, PNB and PNC. Genomic DNAs of the three species were first extracted by using modified method of phenol extraction and ethanol precipitation. Of the 16 random primers tested, only 14 amplified DNA segments (OPA-1, OPA-3-5, OPA-7-10, OPA-12, OPA-14, OPA-16 and OPA-18-20.). From the selected 14 primers, OPA-16 and OPA-18 primers were used for genetic similarity and genetic distance analysis as the bands produced were clear, distinct and reproducible. Genetic similarity and genetic distance of the three species of *Pseudo-nitzschia* was then calculated by using the Nei and Li (1979) equation. The genetic similarity observed between PNA and PNB was 0.5941, followed by PNB and PNC with value 0.5012 and finally PNA and PNC with value 0.4143 while the genetic distance calculated between PNA and PNB was 0.4059, followed by PNB and PNC with value 0.4988 and finally PNA and PNC with value 0.5857. Cluster analysis of distance values was conducted to construct a dendrogram. From the analysis, it was found that the *Pseudo-nitzschia* species; PNA was very much closer to PNB compared to PNC.

ABSTRAK

Dalam kajian ini, teknik RAPD telah digunakan untuk membezakan tiga species alga genus *Pseudo-nitzschia*; PNA, PNB dan PNC. Pengekstrakan DNA bagi ketiga-tiga species ini adalah dilakukan dengan menggunakan pengubahsuaian kaedah pengestrakan fenol dan pemendapan alkohol. Daripada 16 primer rawak yang diuji, hanya 14 megamplifikasi jalur-jalur DNA (OPA-1, OPA-3-5, OPA-7-10, OPA-12, OPA-14, OPA-16, OPA-18-20). Daripada 14 primer-primer yang terpilih ini, primer OPA-16 dan OPA-18 telah digunakan untuk analisis persamaan genetik dan jarak genetic disebabkan jalur-jalur yang dihasilkan adalah tebal, jelas dan dapat dihasilkan semula Persamaan genetik dan jarak genetik bagi ketiga-tiga species *Pseudo-nitzschia* dikira selepas itu dengan menggunakan persamaan Nei dan Li (1979). Persamaan genetik yang didapati antara PNA dan PNB adalah 0.5941 diikuti oleh PNA dan PNB dengan nilai 0.5012 dan akhirnya PNA dan PNC dengan nilai 0.4143 manakala jarak genetik yang dikira antara PNA dan PNB adalah 0.4059 diikuti PNB dan PNC dengan nilai 0.4988 dan akhirnya PNA dan PNC dengan nilai 0.5857. 'Cluster analysis' bagi jarak genetic adalah dijalankan untuk membina 'dendogram'. Daripada analisis data, adalah didapati bahawa *Pseudo-nitzschia* species; PNA adalah lebih dekat kepada PNB berbanding dengan PNC.

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ABBREVIATION

ASP	Amnesic Shellfish Poisoning
CFP	Ciguatera Fish Poisoning
DAP	Domoic Acid Poisoning
dH2O	Distilled water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetate
EtOH	Ethanol
HAB	Harmful Algal Bloom
HCl	Hydrochloric acid
H_2O	Water
NaCl	Sodium Chloride
$NaNO_3$	Sodium nitrate
NSP	Neurotoxic Shellfish Poisoning
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SDS	Sodium dodecylsulfate

NOMENCLATURE

bp	base pair
°C	Degree Celsius
cm	Centimeter
g	Gram
km	Kilometer
µl	Microliter
µm	Micrometer
mg	Milligram
ml	Milliliter
mM	Millimolar
min	Minute
M	Molar
ng	Nanogram
pmol	Picomole
s	Second
u	Unit
W/V	Weight per volume
%	Percent

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Algae are an extremely diverse group of organisms that have important functions in aquatic habitats (Stevenson *et al.*, 1996). Basically, the algae photosynthetic system is based on chlorophyll a and oxygen. Besides chlorophyll a, algae may also possess other chlorophylls pigments, carotenoids and phycobiliproteins in their photosynthetic system depending on the algae divisions (Sze, 1993).

In the developmental stage of algae, there is no forming of embryos within the coverings produced by parents. More over, there are no sterile tissues in algae reproductive structure but all cells are potentially fertile (Sze, 1993). However, these characteristics show variation among species of algae. There are no photosynthetic pigments found in some typical species that are classified as algae (Sze, 1993).

Algae show a broad range of complexity from microscopic single cells to seaweed reaching 30m in length. Besides, they also show variations in their cellular structures, in

their cell arrangement to form multicellular bodies or thalli and also in their photosynthetic pigments (Sze, 1993).

In the marine and freshwater environments, algae continue to be the important producers of oxygen and organic materials. For example, the microscopic planktonic algae function as vital food for the filter-feeding bivalve shellfish such as oyster mussels, scallops, clams as well as the larvae of commercially important crustaceans and finfish. Therefore, the proliferation of planktonic algae in most cases is beneficial to the aquaculture and wild fisheries operations (Hallegraeff *et al.*, 2004).

In some extraordinary situations however, proliferations of microalgae can be very destructive, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts (Hallegraeff *et al.*, 2004). The proliferation of microalgae in this situation is described as Harmful Algal Blooms (HAB).

There are three different types of HAB. The first group of HAB is microalgae that can cause anoxia and indiscriminately kills marine life after reaching dense concentrations. The second group of HAB is microalgae that can contaminate seafood or kill fish massively through food chains and lastly the third group is microalgae that are non-toxic to humans but harmful to fish and invertebrates by damaging and clogging their gills (Hallegraeff *et al.*, 2004). Statistics showed that among the approximately 5,000 recognized species of marine phytoplankton, there are only about 90 species that can

produce potent toxins that can cause negative impacts on fish and human health while only 300 species that can occur in sufficient concentration to discolour the surface of the sea (Bowers *et al.*, 2000).

The significance of discriminating *Pseudo-nitzschia* species is emphasized by the fact that some species *Pseudo-nitzschia* have the ability to produce the potent neuron toxin domoic acid. This toxin can cause serious illness or even death in humans and certain marine birds by contamination of shellfish (Bates, 2000). In this study therefore, RAPD method was developed to discriminate algae genus *Pseudo-nitzschia* to ease the future identification of toxin *Pseudo-nitzschia* species.

This RAPD technique involves PCR amplification with a single random primer to generate a collection of DNA fragments or a fingerprint which is expected to be consistent for the same primer, DNA and conditions used (Asensio *et al.*, 2001). There were 16 random primers used to amplify DNA fragments from PNA which stands for *Pseudo-nitzschia* species A, PNB which stands for *Pseudo-nitzschia* species B and PNC which stands for *Pseudo-nitzschia* species C. These species are hardly discriminated morphologically. The size distribution of amplified fragments varies among species in which closely related species have similar fragment distribution while distantly related ones are more divergent (Prathepa and Baimai, 2002). Thus, RAPD fragments distribution contain considerable phylogenetic information could be used for differentiation at an individual or species level.

1.2 Research objective

The objectives of this research were

1. To obtain RAPD fingerprinting of the three species in the genus *Pseudo-nitzschia* by using random amplified polymorphic DNA (RAPD).
2. To determine the genetic similarity and genetic distance of the three *Pseudo-nitzschia* species.
3. To construct dendrogram of the three species.

CHAPTER 2

LITERATURE REVIEW

2.1 Algae

The algae are not one closely related taxonomic group but a heterogeneous group of organisms which share only a few characteristics (Sze, 1993). Algae are constructed fairly simple whereby they generally do not have vascular tissue, and they do not show high level of organ differentiation of more complex plant. All divisions of algae have chlorophyll a, but different divisions can also have chlorophylls b, c or d. Distinctive accessory pigments such as carotenoids and phycobiliproteins are also characteristics of different algae (Stevenson *et al.*, 1996). Besides, algae have naked reproductive structure and their reproductive structures consist of cells which are potentially fertile.

The algae are divided into different divisions based on their photosynthetic pigments, carbohydrate reserves, flagellum shapes, cell wall components and cell structures (Morris, 1988). The eight divisions are Division Cyanophyta (blue-green algae), Division Prochlorophyta, Division Chlorophyta (green algae), Division Chrysophyta,

Division Rhodophyta (red algae), Division Phyrrophyta (dinoflagellates), Division Cryotophyta and Divison Euglenophyta (Sze, 1993). The algae comprise of both prokaryotic and eukaryotic types. The Cyanobacteria (sometimes called as Cyanophyta or blue-green algae) and Prochlorophyta are both prokaryotic divisions while other algae are eukaryotes. Each major division of algae may contain one or more morphologic types. The seven basic types of morphologic features are flagellated solitary cells, colonies, amoeboid cells, filaments, and parenchymatous thalli (Sze, 1993).

Most of the algae are found in the fresh and salt waters that cover over 70% of the earth surface as members of planktonic and benthic communities. Benthic algae are those that live on or in association with substrata while planktonic algae comprising the phytoplankton community are free floating in the water column (Stevenson *et al.*, 1996). Like any other organisms, the algae require certain conditions for growth. The three major conditions are light, temperature, and the availability for inorganic nutrients (Sze, 1993). Algae which occupy a wide variety of marine and freshwater habitats are the primary producers of oxygen and organic material in aquatic ecosystem (Anton & Wong, 1999). However, dense growths of algae may interfere with human activities.

2.2 Diatom

Bacillariophyceae under the division of Chrysophyta are commonly called as 'Diatoms'. It includes a large number of unicellular and colonial algae which vary greatly in form and size (Rajan, 2001). They exist as solitary cells or as members of colonies which often

joined together by mucilage to form filamentous structure. Their reproduction occurs by ordinary mitotic cell division as well as through the formation of an auxospore by sexual reproduction.

The primary distinguish feature of diatoms are that the cell wall consists of a frustule of silica surrounded by mulaginous material. This opaline or glass frustule is composed of two valves which fits together by a connective zone called girdle. The large valve is called epitheca and the smaller half is known as the hypotheca. Additionally, there are often loops of silica inserted between valves, which are called girdle bands (Sze, 1993). Figure 2.1 shows the diatom frustule of centric diatom and pennate diatom.

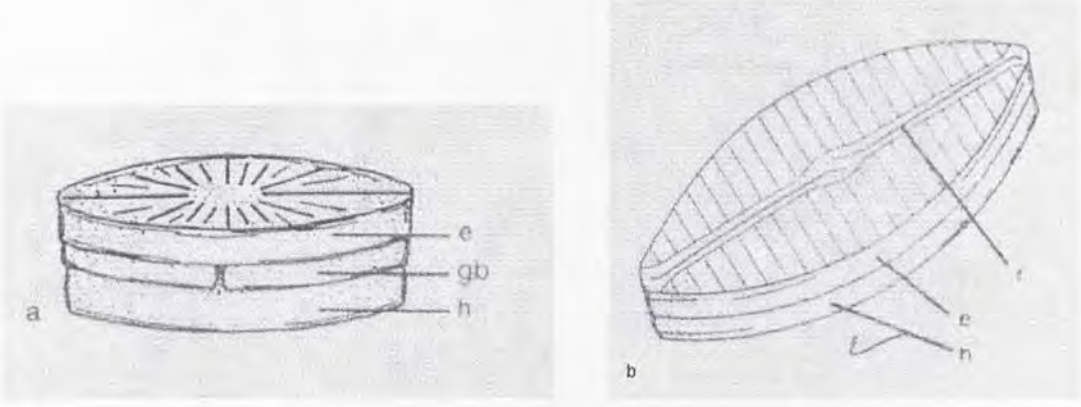

Figure 2.1 Diatom frustule: (a) centric diatom; (b) pennate diatom, e= epitheca, h=hypotheca, gb= girdle band, r=raphe (Sze, 1993).

A diatom cell can be viewed from different angles. Figure 2.2 shows the valve view (view from top) and the girdle view (view from side) of a diatom. There is only one valve can be seen in the valve view. The wall which is known as frustule is silified and made up of pectin. On the wall, costae made of row of dots representing cavities can be

seen extending from the margin but not reaching the centre. The rows of costae are known as striae. A line along the centre of the valve is called raphe and at the centre of the raphe is a welling called central nodule. There are two polar nodules present at both ends of the raphe which allowed mucilage to exude out of the wall and help in locomotion. Inside the silicious cell wall is the cytoplasmic surrounding a large central vacuole. Chromatophores are distributed in the cytoplasm. They have the primary pigment of xanthophylls and carotene. The nucleus is bridge like mass of cytoplasm extends across the vacuole in the centre (Rajan, 2001).

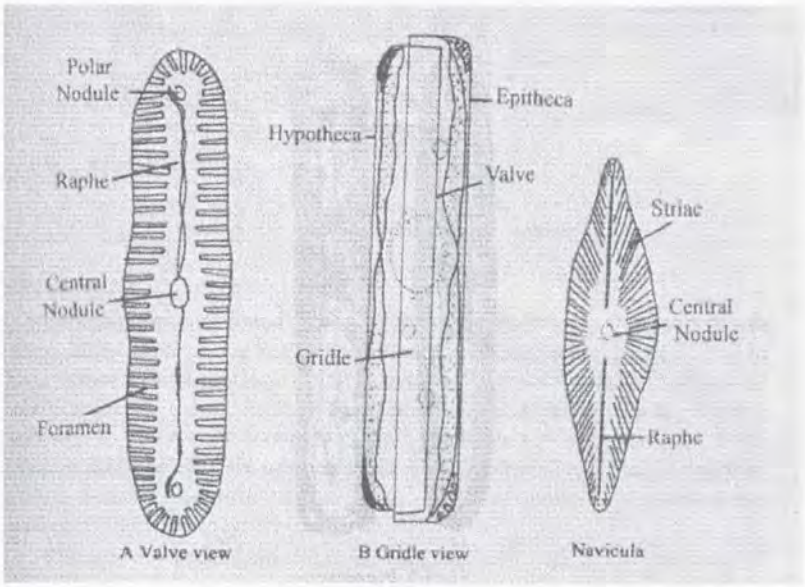

Figure 2.2 Diatoms (valve view and girdle view) (Rajan, 2001).

Diatom is commonly divided into 2 groups, namely Centricae and Pennicae. Centri diatoms are predominantly marine, non-motile diatoms. The shape of these diatoms is equal to a circular pill box which lack of a raphe. It exhibits radial symmetry (symmetry about a point) and has oogamous sexual reproduction. On the other hands,

pennate diatoms are predominately fresh water exhibit locomotion which takes place only in forward and backward direction along the longitudinal axis (Rajan, 2001).

Diatoms are often major components of planktonic and benthic communities in the oceans and freshwaters. Most of the centric diatoms and a few of pennate diatoms are planktonic. Examples of planktonic diatoms are *Coscinodiscus, Planktoniella, Ditylum, Rhizosolenia, Skeletonema, Thalassiosira, Euchampia, Chaetoceros, Stephanodiscus, Nitschia* and *Asterionella*. Diatoms that are known to produce toxins are species of Nitschia and Chaetoceros. Along the east coast of Canada, shellfish have been contaminated with domoic acid from blooms of *Nitschia pungens*. On the other hands, all benthic diatoms are pennate diatoms. Examples of benthic diatoms are *Licmophora* on *Chondrus* and on *Cladophora* (Sze, 1993).

2.3 Genus *Pseudo-nitzschia*

Pseudo-nitzschia is a widely distributed genus of marine planktonic diatoms (Hallegraeff, 1994; Fryxell *et al.*, 1997). In girdle view, this genus is easily identified by the overlapping cell-end tips, which form a step-like chain of spindle-shaped cells. *Pseudo-nitzschia pungens, Pseudo-nitzschia multiseries, Pseudo-nitzschia fraudulenta, Pseudo-nitzschia australis, Pseudo-nitzschia delicatissima* are few examples of Pseudo-*nitzschia* species. However, at present there are no detailed records of *Pseudo-nitzschia* because of the difficulty in discriminating among species using light microscope (Cusack *et al.*, 2004)

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