RIBOSOMAL DNA FINGERPRINTING OF DIATOM, GENUS *PSEUDO-NITZSCHIA*

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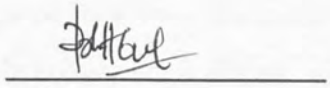

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ABSTRACT

The ribosomal DNA fingerprints of three strains of harmful algae, genus *Pseudo-nitzschia* designated PNA, PNB and PNC were examined. Harmful algae samples were collected from the coastal area of Sepanggar Bay, Sabah. The samples collected were cultured in suspension culture using L1 medium and the algae growth rate were monitored by doing cell counting. PNA reached the log phase after day 13 of culture, while PNB and PNC on day 14 and 16 respectively. Although PNC reached the log phase slower than the other two samples, it recorded more cells during the log phase compared to the other two. When the algae growth rate reached the log phase, DNA was extracted from each of the samples and amplified using primers targeting the small subunit of the rDNA through Polymerase Chain Reaction (PCR). Two PCR products with the size of 1,280 bp and 900 bp were obtained. Subsequently, the PCR products were subjected to restriction enzyme digestions by using *HaeIII*. The digested DNA fragments of the three strains of samples showed that there were genetic differences among the three samples based on the number and size of digested fragments obtained. Four digested DNA fragments were observed in PNA and PNB while six digested fragments were observed in PNC. Degree of similarity calculated according to the Nei & Li (1979) similarity index showed that the degree of similarity between PNA and PNB was 1 and their genetic distance was 0. While the degree of similarity between PNC and these two strains was 0.8 and their genetic distance was 0.2. This showed that PNA and PNB were from the same strain, while PNC was a different strain from PNA and PNB. As a conclusion, in this study genetic molecular technique was found to be useful in identifying differences between algae strains.

ABSTRAK

Pencapjarian ribosomal DNA bagi tiga jenis alga berbahaya daripada genus *Pseudo-nitzschia* yang dilabelkan sebagai PNA, PNB dan PNC telah dikaji. Sampel alga berbahaya telah dikumpulkan daripada pesisiran pantai Sabah. Sampel yang telah dikumpulkan telah dikultur dalam kultur dengan media L1 serta pertumbuhan alga didalam kultur tersebut diperhatikan dengan melakukan pengiraan sel. Didapati bahawa alga PNA akan mencapai fasa log pada hari ke 13 ia dikultur, manakala untuk PNB pula pada hari ke 14 dan hari ke 16 untuk PNC. Walaupun PNC lambat mencapai fasa log, namun ia mencatatkan jumlah sel yang paling banyak ketika dalam fasa tersebut. Apabila pertumbuhan alga mencapai tahap fasa log, DNA ketiga-tiga jenis alga tersebut diekstrak. Kemudian, DNA yang telah diekstrak telah diamplifikasi dalam Tindak balas rantai polymerase (PCR) dengan menggunakan primer yang mensasarkan subunit kecil rDNA. Dua jalur produk PCR yang bersaiz 1280bp dan 900bp telah didapati. Produk PCR yang didapati telah dipotong dengan enzim pembatasan *HaeIII*. Corak pemotongan enzim pembatasan telah menunjukkan bahawa terdapat perbezaan jujukan DNA diantara tiga jenis sampel yang dikaji. empat serpihan DNA didapati daripada PNA dan PNB manakala enam serpihan didapati daripada PNC. Tahap persamaan antara tiga sampel telah dikira dengan menggunakan persamaan Nei dan Li (1979), tahap persamaan antara PNA dan PNB ialah 1 dan jarak genetik mereka ialah 0. Manakala tahap persamaan antara PNC dengan dua jenis sampel tersebut ialah 0.8 dan jarak genetik mereka ialah 0.2 masing-masing. Ini menunjukkan bahawa PNA dan PNB merupakan jenis *Pseudo-nitzschia* sp. yang sama, manakala PNC adalah alga yang tidak sama jenis dengan PNA dan PNB.

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ABBREVIATION

HAB	harmful Algae Bloom
HCl	hydrochloric acid
PCR	polymerase Chain Reaction
Sp	species
Si	silica
ddH_2O	double distilled water
DNA	deoxyribonucleotide acid
SDS	sodium dodecyl sulfate
EDTA	ethylenediamine tetraacetic acid
UV	ultraviolet
PCR	polymerase chain reaction
AFLP	amplified fragment length polymorphism
RFLP	restriction fragment length polymorphism
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
U	unit
dNTPs	deoxyribonucleotide triphosphates
TAE	Tris-Acetate EDTA
A	adenine
T	thymine
G	guanine
C	cytosine

NOMENCLATURE

bp	base pairs
g	gram
µl	microliter
µm	micrometer
°C	degree celsius
%	percent
rpm	revolution per minutes
ml	milliliter
kb	kilobase pairs
mM	milimolar
M	molar
min	minute

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Microalgae are also known as phytoplankton, which is the major food source for fishes and other aquatic living creatures. There are about 30,000 species of algae (Ismail, 1995). Algae serve as the main provider in most of the marine food chain. Therefore, algae bloom should be beneficial to the productivity of the marine ecosystem. However, sometimes algae bloom can cause negative effect to the marine ecosystem and human. This phenomenon is term as harmful algae bloom (Ismail, 1995).

Harmful Algae Bloom (HAB) occurs when a single phytoplankton species is accumulated within a certain places that have a negative affect on the environment (Mudie *et al.*, 2002). Although HAB are usually termed as "red tide", the actual colour of the bloom is actually determined by the pigment of the species blooming (Mudie *et al.*, 2002). Diseases related to HAB are amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP) and ciguatera fish poisoning (CFP) (Mudie *et al.*, 2002).

Diatom is one of the algae which have species that are known to be involve in HAB. Diatoms are unicellular eukaryotes that are photosynthetic, they can exist in simple form or branched, filamentous, and even enveloped in a gelatinous envelope or tube. All diatoms are enclosed by two valves fitted together by a connective zone called a girdle, this structure is known as frustule (MBARI, 1999). A distinctive feature of diatom is its' silica- containing cell wall, that cannot be found in other algae classes (Sze, 1993). Species of diatom reported to cause HAB are the genus *Pseudo-nizschia*(MBARI, 1999).

Pseudo-nitzschia are genus of diatoms that involved in causing amnesic shellfish poisoning (ASP) by producing domoic acid that will accumulate inside marine herbivorous vectors that consume these phytoplankton as their primary food source. These herbivorous vectors will mediate the effect of the toxin on higher organisms that eaten the domoic acid affected vector (Bargu *et al.*, 2003). There have been many known cases of ASP that cause death in both human and marine vertebrates such as the outbreak of amnesic shellfish poisoning in Canada in the year 1989 (Budge and Parrish, 1999).

The occurrence of HAB, which cause human illness and loss of marine life are a major problem for shellfish and fish farming industries throughout the world. Every year, there are about 2000 reported cases of shellfish or fish poisoning and 300 deaths per year globally, with losses to Asian aquaculture industries being as high as US$0.5 billion per year (Mudie *et al.*, 2002).

However, although the impact of harmful alga bloom is serious, we still do not have much information about the mechanism of physiological, behavioral and morphological characteristic of algae (most importantly harmful algae species) interact with the environment. Furthermore, we need a highly effective method to identify harmful alga species during HAB in order to be able to react as fast as possible to reduce the scale of harm that may be caused. The difficulties to identify alga species were one of the main problems in harmful algae bloom research. The conventional methods use in identifying alga is by the observation the algae morphological characteristic. This method did not bring persistent result because some closely related algae species would have almost similar morphological characteristic (Van Dolah, 2000). Therefore, the use of molecular techniques to identify the alga may provide the solution to solve this problem.

In this study, DNA fingerprinting of 3 strains of diatom, genus *Pseudo-nitzschia* were carried out. First, the *Pseudo-nitzschia* was cultured in laboratory. Then DNA was extracted when the *Pseudo-nitzschia* cultures reached mid-exponential phase. The DNA extracted was subjected to polymerase chain reaction using primer targeting small subunit ribosomal DNA. After that, the PCR product was digested by restriction enzyme. The data obtained were analyzed to determine the genetic relationship of the 3 strains of *Pseudo-nitzschia.*

1.2 OBJECTIVES

The morphology of algae had been reported to be varies in different stages of the algae life cycle. The main objective of this study was to compare the ribosomal DNA fingerprint of three strains of *Pseudo-nitzschia* sp. with different morphologies and growth pattern.

CHAPTER 2

LITERATURE REVIEW

2.1 ALGAE

Linnaeus introduced the term "algae" in the year 1754 (Ismail, 1995). Algae consist of about eight divisions of the plant kingdom. Algae are known to be a large and diverse group of eukaryotic organisms that contain chlorophyll and carry out oxygenic photosynthesis (Madigan *et al.*, 2003). In other word, algae can also be described as chlorophyllous, thallophytes and hence autotrophic (Rajan, 2000). Most algae are of microscopic size and so it is consider as microorganism. However, a number of forms of algae are macroscopic, some seaweeds growing to over 30 m in strength (Ismail, 1995). Algae are grouped into 8 groups according to their morphology and pigment. The major groups of algae are Chlorophyta, Euglenophyta, Dinoflagellata, Chrysophyta, Phaoephyta, and Rhodophyta. Algae can be found in freshwater and salt water (Ismail, 1995).

2.2 DIATOMS

Diatoms are also known as the Bacillariophyceae division of algae. It is a microalgae that may exist in pseudofilamentous or colonial form. It contains pigments of fucoxantine, diadinoxanthin and diatoxantin. It stored food in the form of lipid and chrysolaminarin. Diatoms may contain more than one chloroplasts. The most important feature in distinguishing a diatom is that it possesses silica exoskeletons which are known as frustule (South and Whittick, 1987). The frustule consists of two halves, the larger halves are known as epitheca and the smaller halves are known as hypotheca. The are also loops of silica between the two halves which are known as girdle bands (Sze, 1993).

Diatoms are classified into two orders base on the shape of its frustules. The frustules that show radial symmetry are known as Biddulphiales (also called Centrales) while the frustules that show bilateral symmetry are known as Bacillariales (also called Pennales) (South and Whittick, 1987 ; Sze, 1993).

Diatoms carry out both asexual and sexual reproduction. Asexual reproduction is done by binary fission while sexual reproduction is isogamous or anisogamous, with non-flagellate gametes, or oogamous with a uniflagellate male gamete (South and Whittick, 1987). The diatom's cell size will decreased in a percentage of the population over several generation when binary fission is carries out, however, the cell size will be restored when sexual reproduction happened (South and Whittick, 1987; Sze, 1993).

2.3 FACTORS CAUSING DIATOM BLOOM

Studies had shown that diatom blooming usually occurred when water are warm (Shanks and McCulloch, 2003). Diatom blooming is influenced by many factors such as light, nutrients and grazing (South and Whittick, 1987 ; Sze, 1993).

Diatom likes all other algae need light for photosynthesis, on the water surface, light will be sufficient for photosynthesis, in this case, the availability of nutrients becomes the factors that limit the blooming rather than light. Light will decrease exponentially as it penetrates into the water, therefore, diatom only grow well on certain depth in the water (Sze, 1993).

Besides that the presences of nutrients always influence the blooming of diatom and also other algae. However, there are slightly some differences within the nutrient needed by different kinds of algae. For diatom, silicon is one of the major nutrient needed because its silica frustules need silicon in the form of orthosilicic acid (South and Whittick, 1987). During diatom blooming, drastic depletion of silicon in water will happened (Sze, 1993). The other macroelements needed by all algae were carbon, hydrogen, oxygen, sulfur, potassium, calcium, magnesium, phosphorus and nitrogen. While the microelements needed were iron, manganese, copper, zinc, molybdenum and chlorine (Sze, 1993).

Diatom as a primary producer will be consumed by herbivorous animals such as protozoa, rotifers and crustaceans (Sze, 1993). These herbivorous animals will consume or attack algae species that are abundance, thus keeps the algae from blooming. Besides that, grazing also allows for recycling nutrients that sustain high phytoplankton growth rates (Sze, 1993; Bergh *et al.*, 2002).

Diatom bloom is not always beneficial to the marine ecosystem. In some cases, diatom bloom may bring harmful effect. This phenomena is known as harmful algae bloom (HAB).

2.4 HARMFUL ALGAE BLOOM (HAB)

Harmful algae bloom was defined as the proliferations of microalgae in marine or brackish waters that can cause massive fish kills, contaminate seafood with toxin, or alter ecosystem in other ways that can cause harm toward humans (GEOHAB, 1998). The common feature of harmful alga bloom is that they belong to the kingdom of protist (Madigan *et al.*, 2003). Harmful alga bloom is distinguished into two groups which are the toxin producers that can contaminate seafood or kill fish and, the high-biomass producers that cause anoxia and indiscriminate kills of marine life after reaching dense concentration (GEOHAB, 1998). Not all algae in an algae species are harmful. Sometimes, algae within the same species but with different variety may show different harmful characteristic. In other word, a variety of algae may be safe while the other variety of algae within the same species may be harmful (GEOHAB, 1998).

In Malaysia, seafood poisoning caused by harmful alga bloom was first reported in Sabah, and the first case reported outside Sabah was in the early 1991 where three people were poisoned after eating mussels from a mussel farm in Sebatu in the Straits of Malacca (Usup *et al.*, 2002b). The poisoning was suspected to be caused by *P. bahamense*, but to date the species has never been found in plankton samples collected from several locations in the Straits of Malacca. The most recent event of harmful algae related poisoning happened in September 2001, six people were poisoned, including one death, after consuming the benthic clam *Polymesoda* sp. collected from a coastal lagoon in Tumpat on the northeast coast of Peninsula Malaysia. Analysis of clam samples collected from the site during the event using the live mouse bioassay indicated very high levels of alga toxins (Usup *et al.*, 2002b). Algae species from the diatom and dinoflagellate classes are known to be involved in causing Harmful Algae Bloom. Genus *Pseudo-nitzschia* of the diatom class was reported to be involves in causing harmful algae bloom.

2.5 GENUS *PSEUDO-NITZSCHIA*

It is believed that there are over 250 genera of living diatom, which contributed toward 25% of primary production on earth (Scala *et al.*, 2002). However, only a few genus were identified to have harmful algae species. There are 5 species of algae from the genus *Pseudo-nitzschia* were identified to cause harmful algae blooming.

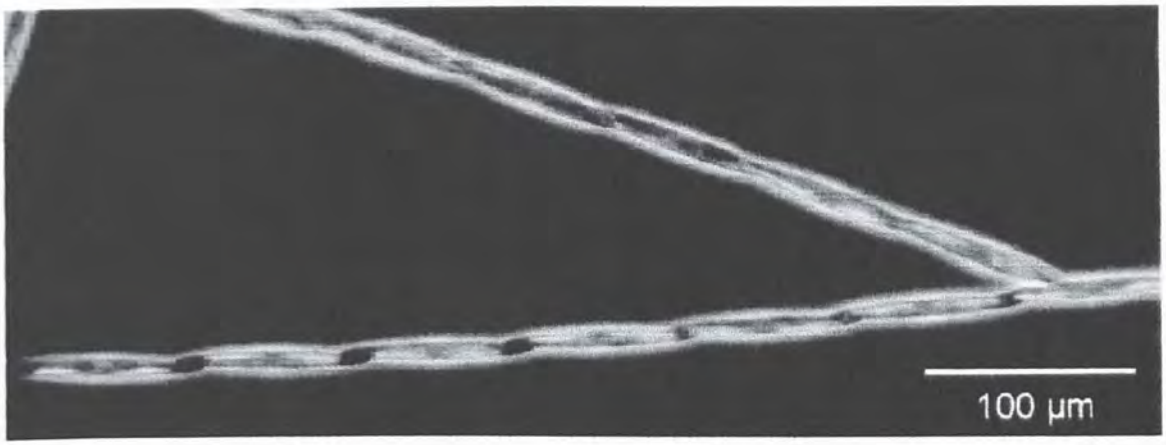

Figure 2.1: Picture of *Pseudo-nitzschia* sp. (Rines, 2002)

Pseudo-nitzschia sp. belongs to the pennate diatom (Bacillariales) groups(Sze, 1993). The genus *Pseudo-nitzschia* (Figure 2.1) originally classified with the genus *Nitzschia* in the past. However, it was separated out from the *Nitzschia* genus because *Nitzschia* have a conopeum near the raphe, and the raphe in a keel rose above the valve surface. In the other hand, *Pseudo-nitzschia* do not had a conopeum near the raphe and its' raphes is not raised in a keel (Hasle, 1993).

Figure 2.2: Structure of *Pseudo-nitzschia* raphe (Shin, 1999).

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