RIBOSOMAL DNA ANALYSIS OF A HARMFUL ALGAL BLOOM (HAB) SPECIES, *PYRODINIUM BAHAMENSE* VAR. *COMPRESSUM* AND ITS ASSOCIATED MARINE MICROBES

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VIVA DATE : **15 JULY 2008**

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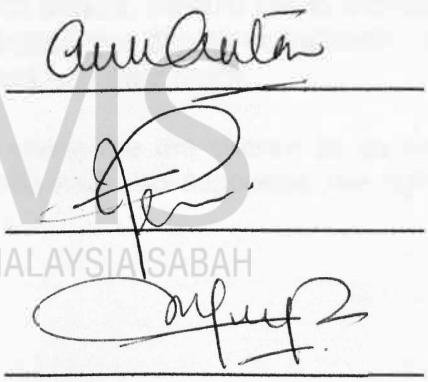

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ABSTRACT

RIBOSOMAL DNA ANALYSIS OF A HARMFUL ALGAL BLOOM (HAB) SPECIES, *PYRODINIUM BAHAMENSE* VAR. *COMPRESSUM* AND ITS ASSOCIATED MARINE MICROBES

Harmful algal blooms in Sabah occur mostly in the coastal waters of west Sabah, where one of the causative organisms is the toxin-producing dinoflagellate, *Pyrodinium bahamense* var. *compressum*. *Pyrodinium* cells were isolated from four locations, namely, Sepanggar Bay, Gaya Bay, Kinarut and Kota Belud and cultured in f/2 medium. Ribosomal DNA fingerprinting of the five *Pyrodinium* isolates was done by means of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the 18S ribosomal DNA. A total of eight restriction enzymes, *Hae*III, *Dpn*II, *Alu*I, *Rsa*I, *Bsa*JI, *Bst*NI, *Bst*UI and *Taq*I, were used to determine the genetic relationship of the five isolates. The RFLP analysis yielded similar restriction patterns among the five isolates. The 18S rDNA sequences of all the *Pyrodinium* isolates were also obtained (Accession numbers: DQ500119 to DQ500123). The size of sequences ranged from between 1,580 to 1,544 base pair (bp). Based on the sequence data, a *Pyrodinium*-specific primer, CWL1R, was designed. To confirm the specificity of this DNA primer, a group of dinoflagellates, such as *Cochlodinium polykrikoides*, *Gymnodinium catenatum*, *Amphidinium* sp., *Gambierdiscus* sp., *Prorocentrum micans*, *Prorocentrum lima*, *Ostreopsis* sp., *Coolia* sp., *Alexandrium minutum*, *Alexandrium tamarense*, *Crypthecodinium cohnii*, *Gonyaulax cochlea*, *Lingulodinium polyedra*, *Peridinium* sp. and *Pyrocystis lunula* were selected for 18S rDNA amplification. The results showed that this primer could only amplify the rDNA of *Pyrodinium*. Therefore, the primer CWL1R has a great potential to be developed as a PCR-based DNA probe for the identification of *Pyrodinium bahamense* var. *compressum*. Sensitivity analysis was also conducted on *Pyrodinium* culture, G1. Based on the results obtained, PCR could amplify DNA template even after the DNA was serially diluted 11 times, where the DNA concentration was 1.6×10^{-9} ng/µl, which correspond to cell concentration of 2.5×10^{-5} cells/l. However, the calculated DNA template concentration for dilution factor 10^{-11} was too low for PCR amplification. This is maybe due to cross-contamination between samples during the dilution process or error in obtaining the DNA concentration in the first undiluted sample. Besides that, ribosomal DNA-based restriction enzyme analysis for the identification of bacteria associated with *Pyrodinium* was also conducted. A total of 16 marine bacterial isolates were successfully obtained from clonal cultures of *Pyrodinium*. The study revealed that all bacterial isolates were Gram negative except for two isolates, which were Gram positive. Restriction enzyme analysis yielded eight different ribotypes. The 16S rDNA sequences of bacteria associated with *Pyrodinium* were obtained (Accession numbers: EF688604 to EF688619). Based on the sequencing results of 16S rDNA, the genetic diversity of the extracellular microbes associated with *Pyrodinium* was limited to the Phyla Proteobacteria and Actinobacteria. The majority of bacteria were *Alcanivorax* sp. and *Hyphomonas* sp., whereas *Kocuria* sp., *Nesterenkonia* sp., *Alteromonas* sp., *Roseobacter* sp., *Xanthomonas* sp., and *Acinetobacter* sp. were identified as minor isolates. These results showed that the dinoflagellate, *Pyrodinium bahamense* var. *compressum* might live, symbiotically or not, with various bacteria in the natural environment.

ABSTRAK

Ledakan mikroalga berbahaya di Sabah kebanyakannya berlaku di perairan barat Sabah, dan salah satu organisma yang bertanggungjawab ialah mikroalga pengeluar-toxin, Pyrodinium bahamense var. compressum. Sel-sel Pyrodinium bahamense var. compressum telah diisolatkan dari empat lokasi iaitu Sepanggar Bay, Gaya Bay, Kinarut dan Kota Belud dan dikulturkan dalam medium f/2. Pengecapjarian Ribosomal DNA (rDNA) kelima-lima isolat Pyrodinium telah dianalisakan dengan menggunakan kaedah polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) berdasarkan 18S rDNA mereka. Sejumlah lapan enzim pembatasan, HaeIII, DpnII, AluI, RsaI, BsaJI, BstNI, BstUI dan TaqI, telah digunakan untuk menentukan pertalian genetik kelima-lima isolat tersebut. Keputusan RFLP menghasilkan corak-corak pembatasan yang sama di antara kelima-lima isolat Prodinium. Jujukan DNA 18S rDNA kesemua Pyrodinium juga telah diperolehi (Accession numbers: DQ500119 hingga DQ500123). Saiz DNA yang telah diperolehi adalah dalam lingkungan 1,580 hingga 1,544 pasangan bes (bp). Berdasarkan data jujukan DNA, sebuah primer spesifik hanya kepada Pyrodinium, CWL1R, telah direka dan kajian populasi juga telah dilakukan dengan beberapa mikroalga, seperti Cochlodinium polykrikoides, Gymnodinium catenatum, Amphidinium sp., Gambierdiscus sp., Prorocentrum micans, Prorocentrum lima, Ostreopsis sp., Coolia sp., Alexandrium minutum, Alexandrium tamarense, Crypthecodinium cohnii, Gonyaulax cochlea, Lingulodinium polyedra, Peridinium sp. dan Pyrocystis lunula untuk menguji spesifikasi primer tersebut. Berdasarkan kajian populasi, primer tersebut didapati spesifik hanya kepada Pyrodinium. Dengan itu, primer CWL1R mempunyai potensi tinggi untuk dijadikan sebagai prob DNA dengan beraplikasikan PCR untuk mengidentifikasi Pyrodinium bahamense var. compressum. Analisis sensitiviti juga telah dilakukan atas kultur Pyrodinium, G1. Berdasarkan keputusan, PCR dapat menggandakan templat DNA yang telah dicairkan 11 kali, ini bersamaan dengan kepekatan DNA 1.6 x 10^{9} ng/µl dan kepekatan sel sebanyak 2.5 x 10^{5} cells/l. Walaubagaimanapun, kepekatan DNA templat pada faktor pencairan 10^{-11} yang dikira adalah sebenarnya tidak dapat digandakan melalui PCR. Ini mungkin disebabkan oleh pencemaran DNA antara sampel-sampel semasa proses pencairan ataupun kesalahan dalam mendapatkan kepekatan DNA pada sampel pertama yang belum dicairkan. Selain itu, analisis enzim pembatasan berdasarkan rDNA untuk identifikasikan bakteria-bakteria berkaitan dengan Pyrodinium juga telah dilakukan. Sejumlah 16 isolat-isolat bakteria telah berjaya diperolehi daripada kultur-kultur Pyrodinium. Kajian awalan telah menunjukkan bahawa kesemua bakteria adalah gram negatif kecuali dua isolat bakteria yang bergram positif. Analisis enzim pembatasan menghasilkan lapan jenis ribotype. Berdasarkan keputusan penjujukan DNA, kepelbagaian genetik bakteria yang berkaitan dengan Pyrodinium adalah daripada Fila Proteobacteria dan Actinobacteria. Bakteria-bakteria utama yang dikenalpasti adalah Alcanivorax sp. dan Hyphomonas sp., manakala Kocuria sp., Nesterenkonia sp., Alteromonas sp., Roseobacter sp., Xanthomonas sp., dan Acinetobacter sp. telah dikenalpasti sebagai isolat-isolat minor. Keputusan ini telah menunjukkan bahawa mikroalga, Pyrodinium bahamense var. compressum hidup, secara simbiosis atau tidak, bersama-sama dengan pelbagai bakteria in persekitaran semulajadinya

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LIST OF ABBREVIATIONS

α	alpha
β	beta
ε	epsilon
γ	gamma
λ	lambda
%	percent
°C	degree Celsius
μg	microgram
μl	microlitre
μm	micrometer
μM	micromolar
ARDRA	amplified ribosomal DNA restriction analysis
AOAC	Association of Analytical Communities
BLASTN	basic local alignment search tool for nucleotide
BSA	bovine serum albumin
bp	base pair
Cl^-	chloride
CTAB	cetyltrimethylammonium bromide
CV	crystal violet
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EBI	European Bioinformatics Institute
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EtBr	ethidium bromide
g	gram
GTX	gonyautoxin
HAB	harmful algal bloom
HPLC	high performance liquid chromatography
h	hour
I	iodine
ITS	internal transcribed spacer
kb	kilobase pair
LD	light dark
LSU	large subunit
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minutes
ml	mililitre
mm	millimeter
mM	millimolar
nm	nanometer

NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NEO	neosaxitoxin
NO_3	nitrate nitrogen
PCR	polymerase chain reaction
pmol	pico molar
PO_4	phosphate
PVP	polyvunylpyrrolidone
RIA	radioimmunoassay
RE	restriction enzyme
RFLP	restriction fragment length polymorphisms
rpm	revolution per minute
rDNA	ribosomal Deoxyribonucleic acid
rRNA	ribosomal Ribonucleic acid
s	second
SSU	small subunit
STX	saxitoxin
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris-HCl EDTA
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
tRNA	transfer ribonucleic acid
U	unit
UV	ultra violet
V	volt
v/v	volume over volume
w/v	weight over volume

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Harmful algal blooms (HAB) are defined as the multiplication or proliferation of plankton algae until they reach high concentrations (usually up to millions of cells per litre) that the surface of the sea becomes discoloured. This phenomenon is also known as "red tides", and can sometimes have negative effects towards aquaculture, fisheries and the tourism industries, as well as environmental and human health. There are approximately 5000 species of extant marine phytoplankton known throughout the world, where around 300 species can cause red tides, while only 80 or so species have the ability to produce potent biotoxins that can be transferred through the marine food chain and eventually end up in humans (Hallegraeff, 2004).

Red tides have become a frequent phenomenon in the coastal waters in many parts of West Sabah. The blooms are caused by a marine dinoflagellate, *Pyrodinium bahamense* var. *compressum*, which was first reported in 1967. This HAB species is an armored, bioluminescent dinoflagellate, and they are the major species involved in the tropical Indo-Pacific red tides (Badylak *et al.*, 2004). *Pyrodinium* blooms are very common in the Southeast Asia region, where the blooms were often reported in the waters of Malaysia (Anton *et al.*, 2000), Brunei (Seliger, 1989), Indonesia (Wiadnyana, 1996) and the Philippines (Azanza-Corrales & Hall, 1993).

The species, *P. bahamense* var. *compressum* produces toxins in shellfish called paralytic shellfish poisoning (PSP) (Anton *et al.*, 2000). PSP in humans is caused by consumption of filter-feeding bivalve shellfish contaminated with a number of saxitoxin derivatives produced by this HAB species (RaLonde, 1996). For this reason, rapid identification and early detection of this PSP toxin-producing species is important before its concentration reaches a level that may give rise to public health problems.

The standard method for detection and enumeration of HAB species in discrete water samples has traditionally been the microscope-based cell identification. Although the traditional method has been reliable, this method is time-consuming and requires expertise to recognize the different key morphological characters of different HAB species (Scholin & Anderson, 1998). The development of molecular or DNA probes can assist in rapid HAB species identification, which allows the early detection of blooms. Probes have the ability to selectively adhere to molecules specifically associated with a particular species or group of species, which then serve as a basis for detecting specific organisms even when they occur in complex natural communities (Scholin *et al.*, 2004).

DNA probes for identifying HAB species commonly target the ribosomal DNA (rDNA) sequence region. The rDNA region is targeted because it is abundant in cells and good recognition is therefore ensured (Rhodes, 1998). The sequences that are potentially unique to the target species are identified and DNA probes directed against those sequences are synthesized (Scholin *et al.*, 2004). In this study, the main focus is to design a DNA probe that is species-specific for the identification of HAB species, *P. bahamense* var. *compressum* infesting the coastal waters of West Sabah.

The triggering action for production of toxins by *P. bahamense* var. *compressum* has been a subject of controversy (Anton *et al.*, 2000). It has been reported that the secretion of toxins during a bloom were not only by the algae themselves but also by the bacteria associated with the toxic algae. Interactions between algae and bacteria are commonly observed in both freshwater and marine ecosystems, where bacteria are postulated to have a major role in regulating the processes of algal bloom initiation, maintenance and also decline (Doucette, 1995; Hold *et al.*, 2001).

Bacteria can live loosely or tightly with the phytoplankton or even live inside the microalgae cells (Córdova *et al.*, 2003; Töbe, 2003). Hold *et al.* (2001) showed that certain bacteria are common to general dinoflagellate cultures, whereas others appear to be specifically unique to a particular dinoflagellate. In this study, the identification of bacteria associated with *P. bahamense* var. *compressum*, can

contribute to future research on bacteria-associated harmful algae by comparing the diversity of bacteria associated with different kind of harmful algae found in the waters of Sabah.

1.2 OBJECTIVES

This study aims to understand the molecular characteristics of *Pyrodinium bahamense* var. *compressum* found in the waters of Sabah based on their ribosomal DNA sequences. The research objectives of this study are as follows:

(a) To compare the ribosomal DNA fingerprints of *Pyrodinium bahamense* var. *compressum* collected from various locations in the Sabah waters by means of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).
(b) To design an oligonucleotide primer that is species-specific for the identification of *Pyrodinium bahamense* var. *compressum* based on the ribosomal DNA sequence.
(c) To identify the bacteria diversity that are extracellularly associated with the toxic algae, *Pyrodinium bahamense* var. *compressum* based on the ribosomal DNA sequence.

1.3 SIGNIFICANCE OF STUDY

The study will identify unique sequences which are species-specific as genetic markers. These ribosomal DNA sequences can be targeted for oligonucleotide probes and subsequently utilized as a tool for the identification of toxin-producing genes.

The identification of *P. bahamense* var. *compressun* using molecular probes will contribute towards the development of a sensitive method for harmful algal bloom detection in Sabah waters. As paralytic shellfish poisoning (PSP) can happen even when cell densities of the *Pyrodinium* are low, the outputs of this study will contribute to the early detection of the presence of *P. bahamense* var. *compressum* even at low densities.

Lastly, this study will contribute to the efficient management of the wild harvest and aqua-culture of shellfish by the responsible monitoring agency, Fisheries

Department of Sabah, in a region where the people are dependent on these resources for their daily consumption and livelihood.

CHAPTER 2

LITERATURE REVIEW

2.1 OVERVIEW OF HARMFUL ALGAL BLOOMS

2.1.1 Harmful Algae

Planktonic algae or phytoplankton are microscopic, single-celled plants that live in the sea. These algae serve as energy provider at the base of the marine food web for filter-feeding bivalve shellfish, such as oysters, scallops and mussels. Occasionally, the algae multiply or 'bloom' until they reach such high concentrations (up to millions of cells per litre) that the surface of the sea becomes discolored (Hallegraeff, 2004). In most cases, the proliferation of planktonic algae is not harmful, but unfortunately in some situations, the blooms are considered harmful because the algae can produce potent natural poisons known as biotoxins that can be transferred through the food web where they affect and even kill, the higher forms of life such as zooplankton, shellfish, fishes, marine mammals and even humans that feed either directly or indirectly on them (Rhodes, 1998).

Blooms of the planktonic algae are commonly referred to as "red-tides", due to the discolorations of the surface water by the pigments of these algae. The term has become a misnomer, however, because blooms can be greenish, yellowish or brownish, and some may not discolor the water at all. Scientists now prefer the term "harmful algal bloom" (HAB) to refer to the bloom phenomenon that produce toxin or that has negative impacts.

Harmful algae can be classified into three different groups, they are: (1) algal species that produce basically harmless water discolorations, however under certain conditions, blooms can grow so dense that they kill non-selectively fishes and invertebrates through oxygen depletion; (2) algal species that produce potent toxins that can be transferred through the food chain to humans, causing a variety of

gastrointestinal and neurological illnesses, such as paralytic shellfish poisoning, diarrhetic shellfish poisoning, amnesic shellfish poisoning, ciguatera fish poisoning, neurotoxic shellfish poisoning and cyanobacterial toxin poisoning; and (3) algal species that are non-toxic to humans but harmful to fishes and invertebrates, especially in the aquaculture industry, by damaging or clogging their gills (Hallegraeff, 2004).

2.1.2 History of Harmful Algal Bloom

The first written reference (1000 B.C.) of HAB was believed to appear in the Bible: '... all the water in the Nile turned to blood. The fish in the river died and the Nile was contaminated so that the Egyptians could no longer drink the water of the Nile. There was blood all over the country of Egypt' (Exodus 7: 20-21). During that time, it was most probably that a non-toxic harmful alga that is harmful to fishes and invertebrates had proliferated in the river, causing death due to oxygen depletion (Hallegraeff, 2004).

Another report of the appearance of HAB was in 1793, where five members of Captain George Vancouver's crew became ill and one died after eating toxin contaminated shellfish collected in Poison Cove on the central British Columbia Coast. Captain George Vancouver had also noticed that the local Indian tribes believed that it was a taboo to eat shellfish when the seawater became bioluminescent by the dinoflagellate blooms. A second shellfish poisoning incident happened in 1799 when about 100 Aleut hunters working for Alexander Baranof died after eating mussels harvested near Sitka, Alaska. In both cases, the shellfish was probably contaminated by a potent toxin called the paralytic shellfish toxins. These toxins accumulate in shellfish and can be fatal to both marine mammals as well as humans (Horner *et al.*, 1997; Hallegraeff, 2004).

2.1.3 Causes of Harmful Algal Blooms

Currently, HABs had apparently increased in their frequency, distributions, severity and variety worldwide. However, causes of the outbreak are still unknown. There are no proven causes, but many highly possible causes. One example of possible causes of the outbreak is the geographical features of the coast (Horner *et al.*, 1997). Horner and his colleague had suggested the water mass of the coastal current of U.S.