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POLYMERASE CHAIN REACTION (PCR) ANALYSIS OF MANGROVE SOIL
MICROORGANISM FROM KOTA KINABALU CITY BIRD SANCTUARY

CLARICE KIAN KOK

THIS THESIS IS PRESENTED TO FULFILL THE REQUIREMENT TO OBTAIN A
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Nama: CLARICE KIAN KOK

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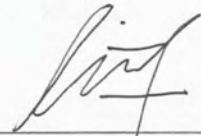
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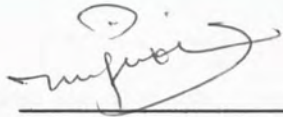
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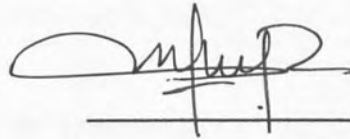
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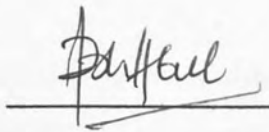
**1. SUPERVISOR
(MS TEOH PEIK LIN)**



**2. EXAMINER 1
(DR. VIJAY KUMAR)**



**3. EXAMINER 2
(DR. ROZIAH HJ. KAMBOL)**



**4. DEAN
(PROF. MADYA DR. AMRAN AHMED)**



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ABSTRACT

The objective of this study was to explore the diversity of the microbial community in soil sample from mangrove soils in Kota Kinabalu City Bird Sanctuary. Soil samples were collected from two different areas that were *Avicenna* (A) and landward (L) in Kota Kinabalu Bird Sanctuary. Two properties of the soil samples specifically pH and distributions of soil particulates were analyzed and this information was imperative in leading us to the type of microorganisms that might be discovered in the respective soil samples. Two direct DNA extraction techniques were employed in the research namely SDS and beat-beating method. From the comparison of two direct extraction methods, the SDS method was a better technique because of the higher quality and quantity of DNA that were obtained. The sample from the SDS based extraction was used in the PCR amplification. The PCR was performed using five set of primers which targeted at specific region of ribosomal DNA (rDNA). From the amplification, sample *Avicenna* contained bacteria and eukaryotes. In the sample of landward, the PCR product was obtained for sample bacteria, *Pseudomonas* and fungus.



ABSTRAK

Objektif kajian ini ialah untuk mengeksploitasikan diversiti bagi komuniti mikroorganisma dalam tanah bakau yang diperolehi dari Kota Kinabalu City Bird Sanctuary. Tanah untuk kajian ini telah diambil dari 2 kawasan dalam perkarangan Kota Kinabalu City Bird Sanctuary iaitu 'Avicenna' (A) dan 'landward' (L). Dua ciri-ciri sampel tanah iaitu pH and pembahagian partikel tanah telah dianalisa and informasi ini adalah penting dalam memberi petunjuk kepada kita kepada jenis mikroorganism yang bakal ditemui dalam sampel tanah berkenaan. Dua kaedah pengestrakan terus telah dipraktikkan dalam kajian ini iaitu cara berdasarkan SDS-Proteinase K and pukulan 'beads'. Daripada perbandingan yang dibuat, didapati cara pengkestrakan berdasarkan SDS-Proteinase K adalah pilihan yang lebih baik berdasarkan kualiti dan kuantiti DNA yang diperolehi. Sampel daripada kaedah SDS ini telah digunakan bagi tujuan amplifikasi PCR. Amplifikasi telah dijalankan menggunakan 5 set 'primers' yang menjadi target dalam kawasan specific ribosomal DNA (rDNA). Hasil daripada amplifikasi ini, didapati sampel tanah dari 'Avicenna' (A) mengandungi bakteria dan eukaryot. Dalam sample 'landward' (L) pula, PCR produk bagi bakteria, *Pseudomonas* dan fungi telah diperolehi.



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LIST OF SYMBOLS AND ABBREVIATIONS

A	Adenine base
ARDRA	Amplified ribosomal DNA-restriction analysis
bp	Base pair
C	Cytosine base
CTAB	Hexadecyltrimethylammonium bromide
°C	Degree Celsius
cm	Centimeter
ddH ₂ O	Double distilled water
dH ₂ O	Distilled water
dNTPs	Deoxysiroenucleotide 5'-triphospahte
EDTA	Ethyladiazine tetraasetic acid
eds	Editors
e.g.	Example
G	Guanine base
g	Gram
HCl	Hydrochloric acid
H ₂ O	Water
Inc.	Incorporated
IGS	Intragenic spacer
ITS	Intraspecic transcribe spacer
kbp	kilobase pair
KCl	Calcium Chloride
LSU	Large subunit
PEG	Polyetheline glycol
μl	Microliter
μM	Macromolar
mg	Milligram
Mg ²⁺	Magnesium ion



MgCl ₂	Magnesium chloride
ml	Millimeter
mM	Milimolar
M	Molar
Na	Natrium
NaCl	Natrium Chloride
ng	Nanogram
O ₂	Oxygen
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
S	Surbeg
s	Second
Sample A	Sample <i>Avicenna</i> (A)
Sample L	Sample landward (L)
SDS	Sodium dodecyl sulfate
sp	species
SSU	Small subunit Universal
T	Thymine base
TAE	Tris-base, Natrium Acetate, Na ₂ EDTA
TE	Tris-EDTA
T _m	Melting temperature
Tris	Tris (hydroxylmetil) aminometena
Tris-HCl	Tris (hydroxylmetil) aminometena- hydroxyl
U	Unit
UV	Ultra-violet
V	Voltage
vol	Volume
w/v	Weight over volume
-	Negative
X	Times
%	Percentage



CHAPTER 1

INTRODUCTION

1.1 Introduction

Microorganisms are small organisms which individually is invisible to the naked-eyes of human. Due to their dwarf sizes, they are distributed almost every part of the environment including soil, sea, air and even some unfamiliar places in the extreme atmosphere that is not suitable for higher living organisms such as the thermal vernal or hot springs. The soil in particular is an interesting environment to study the biodiversity of these microorganisms. In the top 2 cm of mangrove sediment for example, there maybe up to 3.6×10^{11} bacterial cells per gram (dry weight) of sediments and productivity of $5.1 \text{ g carbon m}^{-2} \text{ d}^{-1}$ (Hogarth, 1999).

Although they are small in size, their activities are significantly important in the working of the biosphere (Madigan *et al.*, 2003). Realizing the value of these microorganisms, they have been exploited in a myriad of natural as well as for biotechnological processes. Thus, these microorganisms are extensively been identified



by a variety of methods in the areas such as in the healthcare, food and water quality testing, ecology and so forth. In the health care particularly in pharmaceutical, these microorganisms have been the diagnostic tool in the development of the natural products including clinically important antibiotics such as tetracycline, erythromycin, vancomycin, β -lactams, cephalosporins, and rifampicin (Osburne *et al.*, 2000).

However, due to the minority fraction of the microorganisms that can be cultivated, only a modest understanding of the microorganism diversity was acknowledged. Over 90-99% of microbial cells in the environment that are uncultivable remained to be unstudied (Trevors & Elsas, 1995).

A major breakthrough in the identification and classification of microbial diversity has come with the introduction of useful techniques in determining the specific microorganisms at DNA level. With the utilization of methods are based on the analysis of nucleic acids, more bacteria or microorganisms can be studied from the total DNA isolation of the environment. The PCR based method has greatly accelerate the detection of specific DNA sequences starting from a small number of DNA template. Some of the PCR-based procedures which includes the G+C composition (Nüsslein & Tiedje, 1998; 1999), rDNA (Weisberg *et al.*, 1991, Wilson *et al.*, 1990) and *in situ* hybridization of rRNA oligonucleotide probes makes the study the microbial communities to be possible.

In this research, the microbial diversity in the mangrove soil samples was studied by amplifying specific regions in the nuclear rDNA such as the small subunit and the



intraspecific transcribe (ITS) region. The small subunit genes (16S rDNA and 18S rDNA) were important in the study of bacteria and eukaryote respectively. Drancourt *et al* (2000) has reported that the study of soil community of bacteria was confirmed by using the 16S rDNA genes. The comparisons of nucleotide sequences of rDNA that are highly conserved and that are present in all microorganisms can be also utilized as a means of phylogenetic purposes and to distinguish different microorganisms from the other (Nakatsu, 2000).

The ribosome internal transcribe spacers (ITS) which is the non-transcribe region is another target in the rDNA. Sometimes the limitation for closely related species and strain can be overcome with the analysis of this region. In this research, the ITS region were used for the amplification of eukaryote and fungus. The first step of this research was to perform the bulk DNA extraction from the mangrove soil. The purification of the crude DNA was later done before the polymerase chain reaction (PCR) step was conceded.

The objective of this research was to study the microbial community of the mangrove soil from Kota Kinabalu City Bird Sanctuary by amplifying the specific regions of ribosomal DNA (rDNA) based on polymerase chain reaction method. In this research, different sets of primers were used to target at specific region of ribosomal DNA (rDNA) microorganisms in soil sample. Primer 27f-1525r was used to amplify 16S rDNA of bacteria whereas primer PSuf-PSUr was used to target on bacteria type *Pseudomonas*. Primer FITSf-FITSr was used to target ITS region in fungus. Primer



EITS1f-EITS4r on the other hand was used to target ITS region of eukaryote and primer 18S69f-18S1577r was used to target on the 18S rDNA of eukaryote organisms.



CHAPTER 2

LITERATURE REVIEW

2.1 The tree of life

Over 300 years ago, the molecular phylogenetic study has been compiled in a robust map of evolutionary diversification or known as the tree of life. The evolutionary lives in the world can be distributed among 3 main related domains of Archea, Bacteria and Eucarya. The general properties of these related domains indicate that there is a common ancestor to all living life form. Thus, in the search of common ancestor, more unexpected of evolutionary linkages was discovered especially in the microbial domain.

2.1.1 Microbial diversity

The existence of the microbial life was only acknowledged 3 century ago when the first microscope was invented by Antoni van Leeuwenhoek. The greatest potential in the utilization of biodiversity in biotechnology lies in microbial diversity, but not realized



partly due to its invisibility to the naked eye (Ho, 2003). The soil diversity for instance is one of the environments that more investigation is done. A handful of soil contains billions of microbial organisms, so many different types that accurate numbers remain to be unknown (Pace, 1997)

The exploitation of the microbial diversity enables us to obtain the benefit of the natural products such as the antibacterial, antitoxic, anticoccidial agents, antifungal drugs, herbicidal agents, anticancer drugs, insecticidal and nematocidal agents, immunomodulating compounds, and enzyme inhibitors and so forth. Because of the compromising result the natural products besides in biogeochemical cycling and other ecosystem functions, the microbial diversity, much effort is currently directed at understanding the role of biodiversity in the natural environment.

2.1.2 Microbes producing natural products

Over the time, medicinal chemists sustain an enthusiastic interest in the broad scale of natural products produced by microorganisms like antibiotics and other drugs for the treatment of some serious diseases. Actinomycete is one of the definite microbes which are capable in producing natural products (antibiotics). They are used as antitumor agents, immunosuppressive agents, hypocholesterolemic agents, enzyme inhibitors, antimigraine agents, and antiparasitic agents (Demain, 1999). Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural source, mainly based on their use in traditional medicine (Cragg & Newman, 2001). Effective chemotherapeutic and other bioactive agents can be obtained



from novels of natural products that are based on their biological activities by the use of the combinatorial chemical and biosynthetic technology. In the 1980s, many large pharmaceuticals companies conducted natural products discovery programs (Osburne *et al.*, 2000).

2.1.3 Microbial diversity in soil environments

Soil remains as one of our most precious resources. The realistic thought is that the soil plays an important role in the biochemical cycles, atmospheric controller (Conrad, 1996), filters, remediate anthropogenic pollutant and enables food production (Kennedy & Smith, 1995). One of the most significant components of soil, are the microorganisms.

These soil microorganisms are only capable to be cultivated by using standard method of cultivation. The number of species currently cultivatable from soil is thought to represent only 1% or less of the total population (Osburne *et al.*, 2000). Studies have shown that the soil samples contain the high diversity of bacteria and recently the Archea have occurred to be predominantly in 'extreme' environment.

2.2 Mangrove

Mangroves are the sometimes called rainforests by the sea or the estuaries. It is found in the intertidal zone where the salt water from the sea is brought by the tide. The mangrove



is a unique category of wetland that can be found in tropical climate. "Zonation" often characterizes mangrove forests. (<http://www.ramsar.org>).

Scientists theorize that the earliest mangrove species originated in the Indo-Malayan region (<http://www.ramsar.org>). For this reason, it is clear that there are more species of mangrove in this province. The greatest diversity of mangrove species exists in Southeast Asia (<http://www.ramsar.org>). In Malaysia, there are about 641,000 h.a of mangroves in Malaysia of which 57% are found in Sabah, 26% in Sarawak and the remaining 17% in peninsular Malaysia (Yahaya *et al.*, 2003).

Mangrove is considered very important to mankind as the mangroves help to protect coastlines from erosion, storm damage, and wave action. The mangrove is valuable to mankind productivity such as source of wood, fisheries, medical, food and drink. But the particular uses provided by the mangrove are depending on the nature of the forest (Hogarth, 1999).

Evolutionary adjustments to varying coastal marine environments have produced some astounding biological characteristics within mangrove plant communities. Wetland soils also support immense populations of microorganisms in which some of these microorganism are use pesticides and other organic molecules as food. Bacteria are important in facilitating the breakdown of mangrove litter as well as the cycle of the carbon through the mangrove as a whole (Hogarth, 1999).



2.2.1 Mangrove zones

The mangrove zonations are classified based on the growth of mangrove tree species. The mangrove zonation that usually can be distinguish are the zonation of *Avicenna* (red mangrove), *Sonneratia*, *Rhizophora* (black mangrove), *Bruguire* and further to the inland, landward zone are usually found. In areas of moderate to low rainfall, the landward zone is usually found, especially if the freshwater seepage present. Soils are generally firm, gently sloping and not highly saline. The responses of the mangrove ecosystem are based on the external forces such as the nutrient availability, degree of oxygen deficiency and salinity of soil moisture besides the seedling dispersal, size and growth rate of mangrove plants, and destruction of seedlings by crabs and shade tolerance.

Among the true mangrove species that can be found in Sabah are *Avicenna*, *Sonneratia*, *Rhizophora*, *Bruigera*, *Scyphiphora*, *Lumnitzera*, *Ceriops*, *Cassine* and a few more species (Chim, 1977). But these species are becoming more decreasing because of the pollution and human activities.

2.2.2 The soil microorganisms from mangrove

The soil in mangrove is made of thick organic matter mixed with sediments, and is anaerobic except for the sediment surface (Holguim *et al.*, 2001). The highly productive and diverse microbial communities live in tropical and subtropical mangrove ecosystem. Theses microorganisms are important in the recycling and conservation of nutrients in the



mangrove ecosystem by their involvement in the decomposition of the organic matter. Two main groups these class of decomposition are the bacteria and fungi (Tate III, 2003; Holgiun *et al.*, 2001). These bacteria groups that can be found in the mangrove ecosystem include the nitrogen-cycle bacteria, phosphate-solubilizing bacteria, aerobic photosynthetic bacterial and methanogenic bacteria.

Researches on the mangrove soil have uncovered that an organic solvent tolerant bacteria is a newly discovered group of microorganisms with novel tolerance mechanisms. These searches of bacteria are momentous due to their massive prospective in non-aqueous bio-catalysis, industrial processes involving biphasic organic aqueous fermentation systems, effluent treatment and bioremediation in hydrocarbon-saturated environment (Sardessai & Bhosle, 2002).

2.3 Nuclear ribosomal DNA (rDNA)

Nuclear rDNA gene is present in all cellular organisms that have DNA as their hereditary material except the virus. The rDNA encodes for the cellular organelle that performs protein synthesis. As for that reason, the highly conserved molecular markers and/or gene regions are useful for investigating phylogenetic relationship at higher categorical levels (deep branches of evolutionary history) (Hwang & Kim, 1999). The nuclear rDNA consists of repeated unit of coding genes which are nuclear small subunit (SSU), large subunit (LSU) and 5.8S rDNAs. These coding regions are separated by spacers. The SSU and LSU rDNA are separated from each other by 2 external transcribed spacers (ETS)



and a non-transcribed spacer (NTS). Both of these spacers are frequently called an intergenic spacer (IGS). The internal transcribed spacers (ITS1 and ITS2) can be found implanted between the 5.8S rDNA as seen in Figure 2.1. Due to the useful properties in nuclear ribosomal, this region is an interest of study especially in the evolution, phylogeny, systematics and identification as well as in the field of ecology (Stackebrandt & Rainey, 1995)

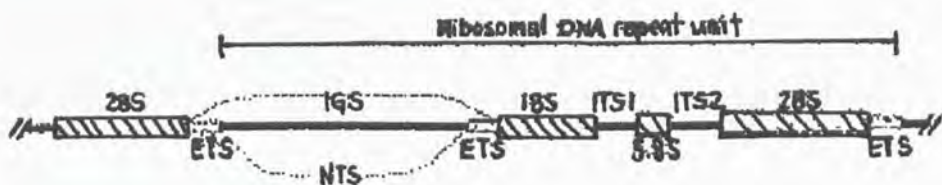


Figure 2.1 A unit of tandem repeat rDNA region (Hwang & Kim, 1999)

The nuclear SSU (16-18S) rDNA is one of the coding regions in nuclear rDNA which is highly conserved. The 16S rDNA which contain 1500nt has been used to construct deep phlogenetic branches including kingdoms, phyla, classes or order (Hwang & Kim, 1999; Stackebrandt & Rainey, 1995; Amann *et al.*, 1995).

The Nuclear LSU (23-28S) is the largest subunit of rDNA and show more variation and thus is more preferential to examine low category of levels such as the class, order of families as reported by the Hwang and Kim (1999). The LSU size for prokaryote

is 23S whereas the eukaryote size of LSU is 28S. It has a variable size of genes due to the different domains or expansion segments.

The ITS regions which can be subdivided as ITS 1 and ITS 2 are another important region in the nuclear rDNA. ITS 1 is a non-coding region between SSU and the 5.8S. The ITS regions evolve fastest among the other regions in nuclear rDNA and may vary among species within a genus or among population (White *et al.*, 1990). Due to variability, the ITS have generally used in the construction of phylogenetic tree in the lower categorical levels among genera, species or population (Hwang & Kim, 1999). The size of the coded by this region is 1 kb as reported by Hwang & Kim (1999).

The IGS are composed of external transcribe spacers (ETS) and a non-transcriber spacer (NTS). The nuclear rDNA spacer regions evolve much faster than the nuclear rDNA coding regions because the substitution occurring in this spacer region do not show lethal effects on the organisms. Thus, it is said that this region is less conserved compared to the coding regions. IGS is used in the construction of lower category among species and population (Hwang & Kim 1999). The size of this region is estimated to be 4-5kb. Despite the large size of this region coded, IGS is not preferred in the study of phylogenetic study in the previous time. But in the later studies of Ryu *et al.* (1999), this region were able to be used in the long PCR technology with the designation of two primers from 28S rDNA 3'-end and 18Sr DNA.



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