METABARCODING OF MITOCHONDRIAL AND CHLOROPLAST DNA ON SELECTED ENDANGERED MAMMALS IN MALAYSIA BY USING NEXT GENERATION SEQUENCING

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This thesis is wholeheartedly dedicated to my beloved parents, Othman and Sharipah and family who have been my pillar of strength throughout my pursuit for PhD

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ABSTRACT

The introduction of Next Generation Sequencing (NGS) has revolutionized the field of wildlife conservation, offering a powerful tool for dietary assessment and gut health analysis in mammals. Previously, most of study focus on the usage of the conventional methods in monitoring of wildlife which require attention from the researches due to the lack of study. Thus, the objectives of the study are to evaluate the diversity of gut microbiomes between wild and captive population of Asian elephant (*Elephas maximus*) using 16S rRNA, characterized the plant consumed by Siamang (*Symphalangus syndactylus*) using trnL marker and characterized the dietary composition between two species of otter, Asian small-clawed otter (*Aonyx cinereus*) and smooth-coated otter (*Lutrogale perspicillata*) using Cytochrome *b* gene. All the outcomes of the research were used as the guideline and reference in developing a framework for next-generation wildlife monitoring in Malaysia based on NGS methods. Our results successfully revealed the identification of 34 phyla, 260 families, and 514 genera in the gut microbiomes of *E. maximus*, with Bacteroidetes (26.8%), Firmicutes (24.2%), and Proteobacteria (23.8%) being the predominant phyla. No significance differences of gut microbiomes was shown in the age and sex of Asian elephant. For diet analysis of Siamang, the sequences were indicated to 97 families and 426 genera where the dominant plant consumed was genus *Ficus* (26.4%). While for otter, the analysis was successfully assigned to four families and 11 species of food consumed by both species. These findings provide important insights into the health status of the Asian elephant, as the gut microbiota is found to be affected by the environmental factors. Additionally, the identification and conservation of plant species consumed by Siamang and food eaten by otter can be easier and more efficient without the need for extensive field observation data. Our study highlights the need for long-term monitoring using fresh faecal samples with NGS, which can complement traditional monitoring methods and enhance biomonitoring efficiency for wildlife especially mammals conservation in Malaysia.

ABSTRAK

Pengenalan Penjujukan Generasi Hadapan (NGS) telah membawa perubahan dalam bidang pemuliharaan hidupan liar terutamanya dalam pemakanan dan analisis kesihatan usus mamalia. Sebelum ini, kebanyakan kajian pemantauan hidupan liar tertumpu kepada kaedah konvensional yang menyebabkan kekurangan kajian, Oleh itu, objektif kajian ini adalah untuk menilai kepelbagaian mikrobiota usus antara populasi liar dan kurungan gajah Asia (*Elephas maximus*) menggunakan 16S rRNA, mencirikan tumbuhan yang dimakan oleh Siamang (*Symphalangus syndactylus*) menggunakan penanda trnL dan mencirikan komposisi pemakanan antara dua spesies memerang, memerang berkuku kecil Asia (*Aonyx cinereus*) dan memerang bersalut licin (*Lutrogale perspicillata*) menggunakan gen Cytochrome *b*. Kesemua hasil penyelidikan telah digunakan sebagai garis panduan dan rujukan dalam membangunkan rangka kerja pemantauan hidupan liar generasi akan datang menggunakan NGS, Hasil kajian ini berjaya menemui 34 filum, 260 keluarga, dan 514 genera dalam mikrobiota usus *E. maximus*, dengan Bacteroidetes (26.8%), Firmicutes (24.2%), dan Proteobacteria (23.8%) menjadi filum dominan. Kepelbagaian mikrobioata usus tidak menunjukkan perbezaan dalam umur dan jantina gajah. Bagi analisis diet Siamang, jujukan menunjukkan kepada 97 keluarga dan 426 genera di mana tumbuhan dominan yang digunakan adalah genus *Ficus* (26.4%). Untuk pemakan memerang, analisis berjaya memenumi empat keluarga dan 11 spesies makanan yang digunakan oleh kedua-dua spesies. Penemuan mikrobiata usus ini disebabkan pengaruh persekitaran yang mempengaruhi kesihatan gajah. Selain itu, pengenalpastian dan pemuliharaan spesies tumbuhan dan haiwan yang dimakan Siamang dan memerang boleh menjadi lebih cekap tanpa memerlukan data pemerhatian lapangan yang meluas. Oleh itu, hasil kajian ini dapat membantu untuk pemantauan jangka panjang menggunakan sampel najis segar yang boleh melengkapkan kaedah pemantauan tradisional dan meningkatkan kecekapan biomonitoring untuk hidupan liar terutamanya pemuliharaan mamalia di Malaysia.

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°C - Degree Celcius
% - Percentage
µg - Microgram
µL - Microliter
µM - Micromolar
bp - Base pair
Cq - Quantification cycle
Cyt*b* - Cytochrome *b*
DNA - Deoxyribonucleic Acid
ddH$_2$O - Deionized water
Gb - Unit for Giga byte
kb - Unit for kilo byte
min - Minutes
mg - Milligram
mL - Milliliter
ng - Nanogram
nm - Nanometer
nM - Nanomolar
OTU - Operational Taxonomic Unit
PCR - Polymerase Chain Reaction
qPCR - Quantitative Polymerase Chain Reaction
RNA - Ribonucleic Acid
rpm - Rotation per minute
sec - Seconds
trnL - Transfer RNA Leucine
WGS - Whole genome sequencing

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CHAPTER 1

INTRODUCTION

1.1 Research background

As one of the 12th top megadiversity countries, Malaysia plays a vital role in conserving thousands of flora and fauna, which can be found throughout the tropical rainforest and have evolved over 130 million years (UNDP, 2018). Malaysia is home to the approximately 361 species of mammals where 223 species found in Peninsular Malaysia (DWNP, 2018), 785 species of birds (MNS, 2015), 654 species of amphibians (Frost, 2023), 506 species of reptiles (Uetz et al., 2022), around 627 species of freshwater fish and 1369 species of marine fishes (Froese and Pauly, 2022). Some iconic species can be found in Malaysia, such as the Malayan tiger (*Panthera tigris jacksoni*), Proboscis monkey (*Nasalis larvatus*), and Sunda pangolin (*Manis javanicus*). However, they are now facing the issue of the declining population due to habitat destruction. Between 2002 and 2020, Malaysia lost more than 2.7 million hectares of humid primary forests, leading to the complicated problems created by deforestation and forest degradation (Omran and Schwarz-Herion, 2020). This issue causes most species to become threatened due to their habitat loss, and some mammals are threatened with extinction listed by the International Union for Conservation of Nature’s Red List of Threatened Species (IUCN Red List). Currently, monitoring wildlife depends on conventional methods like camera traps and wildlife sign surveys to help gather information on species considered time-constraint, especially in getting the fast result to meet the problem arising (Nasron et al., 2019). Establishing a molecular approach through DNA species identification is one of the initiatives that can be implemented in wildlife monitoring (Gani et al., 2019).

The evolution of molecular technology is rapidly increasing with the development of the advanced technology called Next Generation Sequencing (NGS). The introduction of NGS, a high-throughput technology, has been used widely, revolutionizing genomic and genetic diversity (Quail et al., 2012). The significant advance offered by NGS is the ability to provide a massive amount of data that can be applied to understand the evolution and maintenance of biodiversity (Tan et al., 2019). The molecular approach is necessary to assess genetic diversity accelerated due to biodiversity loss and environmental challenges for long-term biodiversity conservation (Hunter et al., 2019). The growth of technologies positively impacts biodiversity due to the ability to explore ecosystem-level processes, conservation dynamics, and the sensitivity for detecting rare species or difficult-to-sample taxa (Tan et al., 2019). The utilization of NGS in wildlife monitoring is increasing to fulfill the demand for conservation efforts as the cost is economical compared to camera trapping, which remains expensive and needs an extended temporal coverage to monitor the wildlife (Burton et al., 2015).

Formerly, molecular approaches using the technique of Sanger sequencing were used in species identification (Abdul-Latiff et al., 2017; Aifat et al., 2016; Md-Zain et al., 2018; Nasihin-Seth et al., 2019). Sanger sequencing is the first-generation sequencing that used even using low-quality DNA (Lalam, 2006). Over the period, second-generation sequencing or NGS was used in ecological and environmental research. From the DNA of higher organisms to smaller organisms, it can be sampled, extracted, and analyzed using the NGS platform (Taberlet et al., 2012a). The platform can provide billions of sequences reads in a single experiment even by using degraded DNA compared with Sanger sequencing, ideally suited for large-scale biodiversity analysis of environmental samples (Shakrolla et al., 2012; Taberlet et al., 2012b). NGS technologies continually improved to become faster, more efficient and cheaper than those used in various fields as mentioned by Slatko, Gardner and Ausubel (2018) and Gupta and Verma (2019). In NGS, a few platforms were introduced, starting with pyrosequencing by Roche sequencing in 2005, Solexa or re-named as Illumina in 2006, SOLiD platform in 2007, and Ion Torrent and Nanopore sequencer that was released in 2011.

Due to its incredible potential, NGS plays significant roles in various analyses of environmental samples, including in wildlife monitoring. In the past, many studies used NGS platforms to identify wildlife's bacterial composition due to the importance of microbiomes in the host (Yao et al., 2019). The relationships between gut microbiomes and wildlife can be linked to various intrinsic and extrinsic factors that can be used to determine animal health as mentioned by Trujillo et al., (2022). The revolution of molecular sequencing can help to study the microbiome in the mammalian gut, which can help clarify the complex relationships between pathogens, microbial diversity and environmental variation (Ficetola et al., 2019). Detection of the gut microbiome from faecal that undergoes through digestion of complex food consumed is also affected by the living environment, specifically in health issues, especially for captive animals (McKenzei et al., 2017; Gibson et al., 2019). The gut information on the bacterial community can help in knowing the mammals' diet preferences that will impact the animals' well-being (Gibson et al., 2019). Therefore, by having DNA sequencing technologies, it will help to develop a broader understanding of the complex microbial communities associated with various habitats influenced by environmental changes.

Furthermore, NGS technology is a promising tool in increasing the understanding of wildlife's nutritional ecology to determine diet, particularly from generalist predators and herbivores where the information is hard to obtain (Pompanon et al., 2012; Liu et al., 2021). The traditional diet observation methods include direct observation, categorized as poor and low resolution, and may lead to the difficulties in studying the diet preferences of wildlife (Mathhews et al. 2020). The importance of identifying the accuracy of dietary analysis of wildlife can be done to evaluate the host health, understand the relationship between animals and the environment, explore predator-prey dynamics, uncover trophic interactions, explain behavioural plasticity and even facilitate pest management (Han et al., 2016). Hence, the comprehensive database to analyze the result from NGS will help the accuracy of the dietary profile, which can be obtained simultaneously (Quemere et al., 2013).

In addition, the DNA metabarcoding introduced by Taberlet et al., (2012a) can be used for a multi-species DNA approach in molecular identification. DNA metabarcoding means species identification of multiple species from a single bulk sample from a single environment through degraded DNA and can provide a rich source of new data in ecosystem monitoring (Baird & Hajibabei, 2012). According to Ji et al. (2013), DNA metabarcoding refers to NGS combined with DNA barcoding, where universal PCR primers are used to amplify the targeted recipient. The effectiveness of NGS can be verified when it is used in analyzing the diets from feces

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VITA

Nursyuhada binti Othman was born on 3rd February 1995 in Alor Setar, Kedah. She received her primary education at Sekolah Kebangsaan Bandar Baru Darulaman. Then, she continued to the secondary school MRSM Kubang Pasu and also MRSM Tun Ghafar Baba after getting straight A’s in PMR. She pursued her undergraduate studies at Universiti Teknologi Mara and her degree at Universiti Malaysia Terengganu. After that, she furthered her education by pursuing a Master of Science in Biodiversity at Universiti Kebangsaan Malaysia in 2019. Currently, she is completing her PhD in Biodiversity at Universiti Tun Hussein Onn Malaysia, where her research is focused on the application of Next Generation Sequencing using Illumina platform on wildlife monitoring by using non-invasive samples. Her research interests also include conservation biology and molecular ecology focused on the wildlife. Nursyuhada has been recognized for her academic achievements with various awards, including the Dean's List Award for her outstanding performance in her undergraduate studies. Upon completion of her PhD, Nursyuhada plans to continue her research in the field of biodiversity, with a focus on developing new techniques for monitoring and conserving wildlife populations especially in Malaysia.