UNIVERSITI PUTRA MALAYSIA

GUT BACTERIAL PROFILING OF MALAYAN TAPIR (Tapirus indicus Desmarest) FROM PENINSULAR MALAYSIA USING 16S rRNA METAGENOMICS APPROACH

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By

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Chairman : Associate Professor Geetha Annavi, PhD
Faculty : Science

Malayan tapir (*Tapirus indicus*) known as one of the endangered species listed by IUCN 2017. Less than 2,000 individuals recorded in the wild environment and the population is at alarming stage. According to field biologists, the main factors lead to tapir population decreasing is deforestation, habitat loss and illegal hunting. Currently, information such as diseases and genetic of Malayan tapirs are still lacking due to their inclusive characteristics and elusive as they lives in deep forest caused obstacles for researchers to collect their biological samples. Fresh invasive sample is required to obtain diseases related information but to collect biological samples of Malayan tapir are challenging due to time constraint, labor intensive and required an expertise. Thus, one of the approaches proposed in this thesis is by collecting faecal samples of tapirs from captivity and performed the study of gut bacteria profiling using metagenomics approach. Guts bacteria profiling acted as "second brain" in tapirs to determine their health condition. The aim of this study is to identify the bacteria profiling using 16S rRNA metagenomics on Illumina MiSeq platform to sequencing the high throughput sequences. Gut bacteria profiling were studied based on intrinsic factors where premeditated thru individual gut bacterial profiles and based on gender category; while extrinsic factors covered thru climate and types of enclosure variants. In nutshell, *Firmicutes* and *Bacteriodetes* were the dominant bacteria phyla founded in this study. Findings from this research has classified into shared bacteria taxanomy between the datasets and exclusively present in singular sub-dataset. From the putative result, we managed to visualised the overview and specific bacteria(s) presented in tapirs based on the intrinsic and extrinsic factors. Interestingly, individuals' gut bacteria profiling were varying and some of the findings provides preliminary information to wildlife management to take precaution actions on the captivity conservation of Malayan tapir. Based on the obtained gut bacteria profiling results, few tapirs involves in this study were probed undergoing obesity, ulcer and vulnerable to water-borne diseases. However, further investigations require such in order to indorse the health condition of tapirs. Emphasizing here that metagenomics application acted as one of the advanced molecular tools only to provide putative bacteria taxonomy but not the meticulous tool to conclude the findings. Thus, other intense animal medical examination test(s) require crossing confirming the health condition of tapirs. The putative findings from this research can be applied as providing primary information to Peninsula wildlife management to diagnosis the diseases or screens the health condition of specific tapir(s), which prone to diseases based on this gut bacteria profile. As well, this study also acts as references for veterinarians to recognise and choose factual individual(s) for captive breeding programs and take care the welfare of the Malayan tapir.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMPROFILAN BAKTERIA DALAM PERUT MALAYAN TAPIR (*Tapirus indicus* Desmarest) SEMENANJUNG MALAYSIA MENGUNAKAN KAEDAH 16S rRNA METAGENOMIK

Oleh

RAMITHA ARUMUGAM

Januari 2019

Pengerusi : Profesor Madya Geetha Annavi, PhD
Fakulti : Sains

Tenuk malayan (*Tapirus indicus*) merupakan salah satu haiwan yang tersenarai dibawah kategori haiwan ancaman oleh IUCN 2017. Kurang daripada 2,000 populasi tenuk dihutan telah direkod dan situasi ini agak membimbangkan. Menurut pakar penyelidik, penebangan hutan yang berlelusan, kehilangan habitat semula jadi dan pemburuan haram merupakan antara punca kekurangan tenuk di hutan. Berdasarkan hasil kajian dan rujukan berkaitan dengan tenuk, informasi genetik dan penyakit adalah sangat terhad. Ia disebabkan oleh ciri inklusif tenuk dan jugak keadaan hutan belukar dimana ia agak sukar diterokai. Kebiasanya, sampel invasif segar diperlukan untuk menjalani penyelidikan berkaitan penyakit akan tetapi untuk mengumpul sampel biologi tenuk Malaysia di hutan belukar adalah agak mencabar kerana kekangan masa, tenaga kerja yang intensif dan memerlukan kepakaran. Oleh itu, aplikasi metagenomik telah dicadangkan untuk mengeksploitasi data berkaitan dengan penyakit melalui mengkaji profil bakteria usus menggunakan sampel najis tenuk daripada pusat pemuliharaan dan zoo. Profil bakteria usus bertindak sebagai "otak kedua" dalam organisma untuk menentukan keadaan kesihatan mereka. Tujuan utama kajian ini adalah untuk mengenal pasti profil bakteria melalui aplikasi metagenomik 16S rRNA dimana instumentasi Illumina MiSeq digunakan untuk penjujukan DNA. Profil bakteria usus dikaji berdasarkan faktor intrinsik iaitu faktor individu dan jantina; manakala faktor extrinsik diliputi oleh faktor iklim dan jenis variasi kandang. Secara ringkasnya, Firmicutes dan Bacteriodetes dikenalpasti sebagai bakteria dominan daripada hasil kajian ini. Penemuan dari kajian ini telah diklasifikasikan dalam dua kategori; bakteria yang dikenalpasti didalam dataset komparatif dan jugak backteria yang hanya hadir dalam sub-dataset tunggal. Dari hasil kajian awal, kami berjaya mevisualisasikan gambaran keseluruhan dan bakteria khusus yang hadir dalam usus tenuk berdasarkan faktor-faktor intrinsik dan extrinsik. Menariknya, profil bakteria usus tenuk adalah berbeza mengikut individu dan beberapa hasil penemuan daripada kajian ini memberikan maklumat asas kepada bahagian pengurusan hidupan liar untuk mengambil langkah berjaga-jaga terhadap pemuliharaan tawanan tenuk Malaysia. Berdasarkan hasil profil bakteria yang

diperolehi, segelintir individual tenuk yang terlibat dalam kajian ini kemungkinan mengalami obesiti, ulser dan terdedah kepada penyakit penularan air. Walau bagaimanapun, kajian lanjut diperlukan untuk mengesahkan keadaan kesihatan tenuk. Menekankan bahawa aplikasi metagenomik hanya bertindak sebagai salah satu alat molekul canggih untuk mentafsirkan taksonomi bakteria sahaja tetapi bukan kaedah yang specifik untuk membuat keputusan muktamat. Oleh itu, ujian perubatan haiwan yang spesifik untuk mengesahkan keadaan kesihatan tenuk dicadangkan. Penemuan dari penyelidikan ini boleh digunakan sebagai maklumat asas kepada pengurusan hidupan liar di Semenanjung Malaysia untuk mendiagnosis penyakit atau mengesan keadaan kesihatan tenuk tertentu, terutamanya yang terdedah kepada penyakit berdasarkan profil bakteria usus. Selain itu, kajian ini juga bertindak sebagai rujukan untuk doktor veterinar untuk mengenali dan memilih tenuk yang sesuai untuk program pembiakan tawanan dan menjaga kesejahteraan tenuk Malaysia.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 11 March 2021

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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ug	Microgram
kg	kilogram
m	meter
km	kilometer
%	percentage
HTS	high-throughput sequencing
NGS	Next Generation Sequencing (NGS)
bp	Base pair
rRNA	Ribosomal ribonucleic acid
ITS	Internal Transcribed Spacer
DWNP	Department of Wildlife and National Parks
a.m	Ante meridiem
p.m	Post meridiem
mL	mililitre
g	gram
C	Celsius
µL	Microliter
nM	Nanomolar
NA	Not applicable

CHAPTER 1

INTRODUCTION

The Malayan tapir (*Tapirus indicus*), also known as the Asian tapir or tenuk in Bahasa Malaysia are important species to the biodiversity conservation of the tropical ecosystem. Apparently, tapirs recognised as living fossil mammal as this genus lives in earth since Eogene span (Janis, 1984). Malayan tapir belongs to the Perissodactyls order, Tapiridae family and share the similar physiology, behaviour, and genetics characteristics with Equidae and Rhinocerotidae families (Medici *et al.*, 2008). Geographically, Malayan tapir scattered around Southeast Asia region including Peninsular Malaysia, South Thailand, Sumatra Indonesia and Myanmar. In the year 2014, the International Union for Conservation of Nature (IUCN) has conducted a survey to estimated Malayan tapirs' population and their presence at Southeast Asia and the data was published in year 2016. Malayan tapirs' population was estimated approximately 2,499 mature tapirs in the wild environment (primary or/and secondary forest, plantation) (Traeholt *et al.*, 2016) and their extant are recorded at South Thailand, Myanmar, Peninsula Malaysia and Sumatra (Indonesia). Human activities such as habitat destruction, plantation transformation and illegal logging (Clements *et al.*, 2012; Traeholt *et al.*, 2016) cause the major declination of tapir population in Southeast Asia. The estimated figure leads to the declaration of Malayan tapir species as "endangered species".

Based on the literatures, information's (Figure 1.0) such as anatomy (Lilia *et al.*, 2010), taxonomy (Medici *et al.*, 2008), habitat preferences and adaptability (García *et al.*, 2012) and distribution of Malayan tapirs (Clements *et al.*, 2012) studied and reported meanwhile other information's like diseases, bacterial infection and genetic studies were lacking (Mangini, Medici, & Fernandes-Santos, 2012). Bacterial infection and diseases information related to Malayan tapir are essential as there are no evidences has reported or proven the population number of Malayan tapirs decreasing due to these factors. Genetic information importance at another hand to discovery the precise information about the present and past demographic parameters, phylogenetic issues, the molecular basis for inbreeding, understanding genetic diseases, and detecting hybridization/introgression in organisms (Michael , *et al.*, 2012). Comparing to the other four sister species which is Lowland tapir (*Tapirus terrestris*); Baird's tapir (*Tapirus bairdii*); Mountain tapir (*Tapirus pinchaque*) and Kabomani tapir (*Tapirus kabomani*) (Medici *et al.*, 2008; Cozzuol *et al.*, 2013) of Malayan tapir, the many information or studies related to this species are lacking behind. To date, cases and studies related to bacterial infection in Malayan tapir are limited. The common bacterial infections reported in Malayan tapirs are Mycobacterium infection and tapirs are infected as the infection spread from other mammal species whom shared the same captivity (Michel, Venter, Espie, & Coetzee, 2003; Schoenbaum, Antognoli, & Orloski, 2014). The consequence of this bacterial infection is it causes sick and death to tapir. Other unreported case occurred in Peninsula Malaysia was sudden death of tapir or serious illness (Yurkovetskiy, et al 2013) during Malayan tapir conservation program (Cheng Li, 2010).

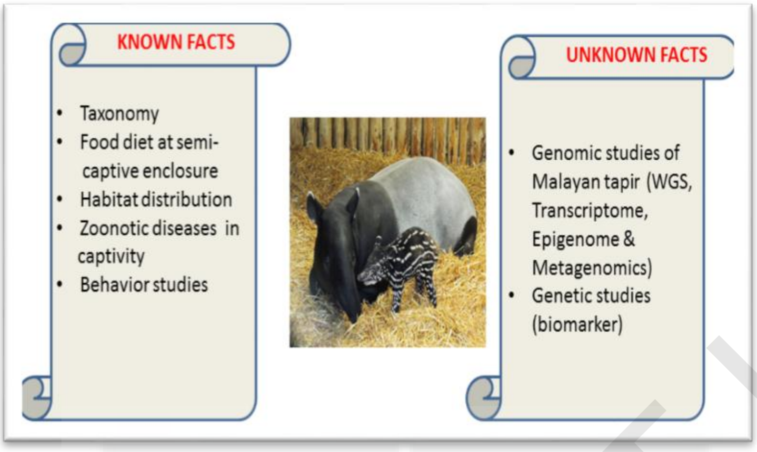

Figure 1.1 : Known and unknown facts about Malayan tapir
(Diagram: Ramitha Arumugam, 2019)

Putative factors lead for lack of studies reported about Malayan tapir possibly due to the factors such as accessibility of invasive and non- invasive samples of Malayan tapir in nature, the sampling process (condition and freshness of samples) (Leendertz, *et al.*, 2006) and transportation of samples to laboratory for downstream process. In additional, to detect the presence the Malayan tapir in nature environment itself very much challenging as there are elusive, shy mammal and immense in camouflage to avoid the contact of prey (Rayan *et al.*, 2012).

Apart of accessing and obtaining the invasive and non-invasive samples of Malayan tapir, there is limitation in detection and annotation of bacteria species diversity. It is due to the molecular biology methods or protocols generally used in detection or/and study the microbes in animals' eukaryotes consisted limitations. Limitations such as growing the culture or microbes in artificial condition are grave challenging as not every microbe's species able to grow in such condition (Spratt, 2004). The limitation of studying in depth about microbes, which is diversity and functional knowledge, remain lean. Many acute and chronic zoonotic diseases cause by microbes yet scientists facing difficulty in distinguishing the bacteria in term of biochemical property and functions (Kosoy, 2013). However, the technology advancement in life science able to overcome the identification of bacteria up to sub-species level and it known as “metagenomics” (Mardis, 2008; Kwon & Ricke, 2011) under Next-Generation Sequencing (NGS) (Attia & Saeed, 2015).

Metagenomics eventually was used to study microorganism abundance in the environmental samples such as aquatic and soil samples (Tringe & Rubin, 2005; Escobar-Zepeda, León, & Sanchez-Flores, 2015). Then, this approach began to evaluate microbiota population in the living organism using biological samples such as stool, urine and blood. The success rate of detecting microorganism using metagenomics approach is accurate up to 99% and it is quite reliable, and helps to answer many unsolved problems related to microorganisms' interaction (Handelsman, 2005). Consequently, this approach has slowly ventured in wildlife animal studies (Poinar, 2006; Thomsen & Willerslev, 2015), for examples in White Rhinoceros (*Ceratotherium simum)* (Bian, Ma, Su, & Zhu, 2013), Red Panda (*Ailurus fulgens)* (Kong *et al.*, 2014) and Giant Panda (*Ailuropoda melanoleuca)* (Zhu, Wu, Dai, Zhang, & Wei, 2011). The potential of metagenomics approach in wildlife study has been proven in the assessment of the physiological condition of experimental organisms, investigative the response of multiple stressors, pH level, nutrients and temperature level in endosymbiont community of aquatic living organism such as corals (Waits & Paetkau, 2005). Metagenomics approach also has a breakthrough the inflammatory bowel disease in mammals (Kostic, Xavier, & Gevers, 2014) and applied to access the starvation stress of wildlife species (Xia *et al.*, 2014; (Thornhill, Howells, Wham, Steury, & Santos, 2017).

Malayan tapirs are presently listed as protected animals under the Wildlife Protection Act 1974, whereby capture and killing of the tapir are not permitted without prior and legal permission from Department of Wildlife and National Parks (DWNP). This Act is applied in research field too. Thus, a suitable research design developed without stressing the Malayan tapir especially during the process collection of sample(s). Non-invasive approach was applied in this research whereby the fresh faecal samples of Malayan tapir were collected and upstream and downstream metagenomics analysis was performed. The gut bacteria profiles derived from the metagenomics analysis explained the bacteria diversity and their symbiosis effect to Malayan tapir involves in this research project.

1.1 Problem statement

As per discussed in the previous paragraph, the limitations in the Malayan tapir lead to this research project. The main idea of this PhD project is to defeat one of the lacking information in Malayan tapirs' research, which is exploration of gut microbiome profiling in Malayan tapir. In this research, obtaining the fresh faecal samples from wild Malayan tapir from primary or secondary forest from Malaysia was constrained. It is due to the to time constraint and man-power constituents. Therefore, this research designed and focussed on the Malayan tapir from captive environment(s) at Peninsular Malaysia. Research finding and output from this project expected to be the guideline for Malayan tapir conservation program in Malaysia. The specific findings in this research were profiling the microbiome taxonomy of each Malayan tapir individual based on four different aspects (individual, gender, climate and enclosure). Findings from this research project will provide a knowledge-based platform whereby, the conservation management able to restructure or modify the conservation program of tapir such as food diet, additional features like providing or built a closed system room for tapirs during wet climate if necessary and et cetera.

1.2 Objectives

The general objective of this study was to profile the total bacterial diversity in Malayan tapir using 16S rRNA metagenomics approach from Peninsular Malaysia to explore the difference in the gut bacteria profiles among the Malayan tapir individuals involved in this study. The specific objectives of this research project breakdown into four (4) based on intrinsic and extrinsic factors states as below:

1. To identify the gut bacteria diversity in eight Malayan tapirs from Peninsular Malaysia using 16s rRNA metagenomics approach.
2. To identify and compare metagenomics of the gut bacteria diversity in Malayan tapir based on gender.
3. To identify and compare gut bacteria diversity in Malayan tapir during two different climates in Malaysia.
4. To identify and compare gut bacteria profiling of Malayan tapir from captive and semi-captive enclosures.

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