PROTEIN ANTI-MICROBIAL POTENTIAL OF THE SKIN SECRETION OF HUMAN COMMENSAL AND FOREST EDGE FROG (ANURAN) SPECIES FROM LANGKAWI ARCHIPELAGO

DASI ONG BINTI YAQUT ONG

UNIVERSITI SAINS MALAYSIA

2021

PROTEIN ANTI-MICROBIAL POTENTIAL OF THE SKIN SECRETION OF HUMAN COMMENSAL AND FOREST EDGE FROG (ANURAN) SPECIES FROM LANGKAWI ARCHIPELAGO

by

DASI ONG BINTI YAQUT ONG

Thesis submitted in fulfilment of the requirements for the degree of Master of Science

September 2021

ACKNOWLEDGEMENT

First of all, my most tremendous praise is to Allah the Almighty for giving me the blessing, strength, and patience in completing this thesis. I want to thank Universiti Sains Malaysia (USM), School of Pharmaceutical Sciences, and Analytical Biochemical Research Center for this chance and opportunity for this research. Special thanks to my respected supervisors, Dr. Shahriza Shahrudin and Associate Professor Dr. Mohd Nazri Ismail, for their guidance, bits of advice, criticisms, and motivations throughout the completion of this research.

I wanted to thank Mr. Mohd Afiq Hazlami Habib, for his assistance and guidance, during proteomics lab work. I wanted to thank my father, Mr. Ong Chiang Gaik, for his time and commitment to assist me during fieldwork. My special thanks to the animal ethics committee of USM for granting permission for this research. My most profound appreciation for the help and guidance from Langkawi Research Centre (PPL), UKM, and the Department of Geopark, Langkawi Development Authority (LADA), for their support along with the research. The research grants used were 1001/PFARMASI/8011004 and 203/CABR/6711674. At the same time, USM Fellowship sponsored my studentship for the last five semesters.

Most of all, I would like to thank both of my beloved parents, Mr. Ong Chiang Gaik and Madam Roziah Mat Akob, not to forget my sister, Dyna Ong, and my aunt Madam Zuraidah Zainal Abidin. Who always supports me throughout my journey in this project, both materially and spiritually. Finally, I would like to express my gratitude to all my friends and Langkawi Island citizen for completing this thesis. Lastly, I would like to acknowledge the examination panel, Associate Professor Nik Fadzly Nik Rosely, Associate Professor Dr. Ramlah Zainudin and Dr. Aidiahmad Dewa.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

3D	3 Dimension
ACN	Acetonitrile
AES	Acoustic Encounter Survey
AMP	Antimicrobial Peptide
AMR	Antimicrobial Resistant Microbes
APD or APD3	Antimicrobial Peptide Database
BSA	Bovine Serum Albumin
CID	Collision-Induced Dissociation
DAF	Dispersion Accounted For
DBAASP	Antimicrobial Peptide Activity and Structure Database
DNH_2O	Deionised Water
DTT	Dithioerythritol
FA	Formic Acid
FCD	Final Coordination Dimension
GO	Gene Ontology
GPS	Global Positioning System
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HW	Head Width
IAA	Iodoacetamide
ID	Identification
IPS	Institut Pengajian Siswazah

LADA	Langkawi Development Authority
LC-MS	Liquid Chromatography-Mass Spectrometry
LRCHC	Langkawi Research Centre of Herpetofauna Collection
MADA	Muda Agricultural Development Authority
MALDI-TOF/MS	Matrix-Assisted Laser Desorption - Ionisation-Time of Flight Mass Spectrometry
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyBIS	Malaysia Biodiversity Information System
NMDS	Non-Metric Multidimensional Scaling
PAST	Paleontological Statistics
PDB	Protein Data Bank Biochemistry, Bioinformatics, Chemistry
PMSF	Phenylmethylsulfonyl Fluoride
PPL	Langkawi Research Centre
PTM	Post-Translation Modification
Q-TOF/MS	Quadrupole Time-Of-Flight Mass Spectrometry
SVL	Snout-Vent Length
TL	Tibia Length
UKM	Univerisiti Kebangsaan Malaysia
UniProt	Universal Protein Resource
UniProt KB	Universal Protein Resource Database
USM	Universiti Sains Malaysia
VES	Visual Encounter Survey
WHO	World Health Organization
WT	Weight

LIST OF APPENDICES

POTENSI PROTEIN ANTI-MIKROB DALAM LENDIRAN KULIT SPESIES KATAK (ANURAN) KOMENSAL DENGAN MANUSIA DAN PINGGIR HUTAN DARI KEPULAUAN LANGKAWI

ABSTRAK

Amfibia merupakan haiwan vertebrata yang paling pelbagai di darat. Ia memainkan peranan yang sangat penting dalam pemuliharaan alam sekitar kerana ia merupakan penunjuk biologi. Selain daripada itu, katak juga adalah salah satu sumber potensi sebatian bioaktif untuk aplikasi farmaseutikal dan bioperubatan. Sebanyak 24 spesies amfibia dari enam famili telah didokumentasikan di Kepulauan Langkawi. Mengikut keluk pengumpulan spesies '*rarefaction'* dengan keyakinan 95%, spesies amfibia di Kepulauan Langkawi telah mencapai dataran tinggi, dan ini menunjukkan bahawa dengan usaha yang dilaburkan semua spesies telah ditemui dalam kajian ini. Berbanding dengan kajian yang telah dilakukan sebelumnya, spesies yang tidak dijumpai dalam kajian ini adalah *Ingerophrynus parvus*, *Limnonectes malesianus*, *Occidozyga lima*, *Raorchestes parvulus*, *Rhacophorus bipunctatus*, dan *Amolops larutensis*. Tambahan lagi, ada spesies tambahan yang dicatat iaitu *Pulchrana laterimaculata*. Analisis penskalaan '*non-multi-dimensional*' mengkategorikan 24 spesies amfibia ini kepada lapan kumpulan yang berbeza mengikut pilihan habitat dan pilihan hutan mereka. Untuk analisis komposisi protein dan peptida antimikrob, individu dewasa bersaiz sederhana daripada setiap spesies- *Odorrana hosii*, *Pulchrana glandulosa*, *Chalcorana labialis*, dan *Hylarana erythraea*, ditangkap dari habitat semula jadi mereka. Rembesan kulit katak ini dikumpulkan, diekstrak, dan dianalisa sebatian proteinnya menggunakan kromatografi cecair-spektrometri jisim (LC-MS). Kepekatan keseluruhan protein *Chalcorana labialis* ialah 0.0174 mg / mL; *Odorrana*

hosii ialah 0.0783 mg / mL; *Hylarana erythraea* ialah 0.0179 mg / mL; *Pulchrana glandulosa* adalah 0.0146 mg / mL. Berdasarkan pangkalan data UniProt (Anura), 49 protein telah dikenal pasti dari *Chalcorana labialis*, diikuti oleh 46 protein di *Pulchrana glandulosa*, 33 protein di *Odorrana hosii*, dan 24 protein di *Hylarana erythraea*. Pada masa yang sama, dengan menggunakan pangkalan data peptida antimikrob, peptida antimikrob dari 11 keluarga yang berbeza telah dikenalpasti dari *Hylarana erythraea*, diikuti oleh sembilan dari *Odorrana hosii*, lapan keluarga di *Pulchrana glandulosa*, dan tujuh keluarga dari *Chalcorana labialis*. *Hylarana erythraea* mempunyai bilangan peptida antimikrob dan kepelbagaian keluarga tertinggi. Ini adalah kerana rembesan kulit *Hylarana erythraea* untuk kajian ini telah dikumpulkan dari kawasan yang mempunyai tekanan persekitaran yang tinggi dan merupakan spesies komensal manusia. Ini bermaksud spesies ini telah menghadapi lebih banyak tekanan dari persekitaraannya berbanding spesies lain. Selanjutnya, potensi sifat bioperubatan yang dikesan dalam katak-katak termasuk perencatan terhadap sel barah dan sel tumor, meminimumkan risiko diabetes (jenis 2), tindak balas terhadap radang, pembekuan darah, mengimbangi homeostasis, aktiviti antimikroba, penyembuhan luka, dan anti-virus (HIV). Sementara AMP memiliki sifat anti-bakteria (termasuk mikroba AMR), anti-kulat, anti-barah, anti-oksidan, anti-virus, anti-parasit (Leishmania), dan penyembuhan luka.

PROTEIN ANTI-MICROBIAL POTENTIAL OF THE SKIN SECRETION OF HUMAN COMMENSAL AND FOREST EDGE FROG (ANURAN) SPECIES FROM LANGKAWI ARCHIPELAGO

ABSTRACT

Amphibians are the most diverse terrestrial vertebrates and are essential for conserving the environment since they act as biological indicators. Furthermore, frogs were one of the primary sources of bioactive compounds for biomedical and pharmaceutical applications. A total of 24 species of amphibians from six families were documented within Langkawi Archipelago. The 95% confidence rarefaction species accumulation curve of amphibian species within the Langkawi Archipelago reaches a plateau, shows that all species are found in this study with the effort invested. Compared to previous studies, the absent species in this study were *Ingerophrynus parvus*, *Limnonectes malesianus*, *Occidozyga lima*, *Raorchestes parvulus*, *Rhacophorus bipunctatus*, and *Amolops larutensis*. Moreover, an additional species was recorded- *Pulchrana laterimaculata*. Non-Multi-Dimensional Scaling analysis of amphibian habitat assemblage categorised these 24 species into eight groups according to their habitat preference and forest assemblage. For protein anti-microbial composition analysis, a medium-sized adult individual of each species- *Odorrana hosii*, *Pulchrana glandulosa*, *Chalcorana labialis*, and *Hylarana erythraea*, were collected from their natural habitat. Using liquid chromatography-mass spectrometry (LC-MS), these frogs' skin secretions were collected, extracted, and analysed for their protein compounds using liquid chromatography-mass spectrometry (LC-MS). The total protein concentration of *Chalcorana labialis* was 0.0174 mg/mL; *Odorrana hosii* was 0.0783 mg/mL; *Hylarana erythraea* was 0.0179 mg/mL; *Pulchrana glandulosa*

was 0.0146 mg/mL. Based on the UniProt (Anura) database, 49 proteins had been identified from *Chalcorana labialis*, followed by 46 proteins in *Pulchrana glandulosa*, 33 proteins in *Odorrana hosii*, and 24 proteins in *Hylarana erythraea*. At the same time, using anti-microbial peptide databases, anti-microbial peptides from 11 different families were identified from *Hylarana erythraea*, followed by nine from *Odorrana hosii*, eight families in *Pulchrana glandulosa*, and seven families from *Chalcorana labialis*. *Hylarana erythraea* have the highest anti-microbial peptide number and family diversity. This is because the skin secretion of *Hylarana erythraea* for this study was collected from an area with increased environmental stress and is a human commensal species. This means this species encounters more habitat stressors compared to other species. Furthermore, the potential biomedical properties detected include the inhibition against cancer cells and tumour cells, minimise diabetes (type 2) risk, response towards inflammatory, blood coagulation, regulate homeostasis, anti-microbial activities, wound healing, and anti-viral (HIV). While AMP possesses anti-bacterial (including AMR microbes), anti-fungal, anti-cancerous, anti-oxidant, anti-viral, anti-parasitic (Leishmania), and wound healing properties.

CHAPTER 1

INTRODUCTION

1.1 Background of the research

The United Nations estimated that at least 40% of amphibian species are facing an alarming extinction rate. The threats that lead to the extinction and decline of these amphibians are habitat loss (Cushman, 2006; Hillers et al., 2008), anthropogenic disturbances (Gillespie et al., 2015), infectious disease (Collins, 2010), pollutions, climate changes, invasive pests, overharvesting in food and pet trades (Gilbert et al., 2012). Amphibians are essential to regulate prey and predator population in the food chain/web (Ives et al., 2005); a crucial biological indicator (Quaranta et al., 2009; Van Meter, 2018); biological control (DuRant & Hopkins, 2008) have significant ecotourism value (Loubser et al., 2001; Balmford et al., 2015); environmental conservation (Dodd & Barichivich, 2007; West, 2018); food source; medical and pharmaceutical properties (Govender et al., 2011).

Biodiversity is the variety/diversity of living organisms, including plants, animals, and microorganisms, within a natural system/ecosystem (Rawat & Agarwal, 2015). While island biodiversity refers to the variety/diversity of living organism that occurs/exist on the island. Biodiversity was explored through the pattern of species richness and distribution (McCain, 2007). Island biodiversity shows higher endemism and risk for loss of diversity than the mainland (Guo, 2021). The high endemism is due to long-term biogeographical isolation, contributing to the small population's minimal gene dilution. That will affect the internal selection and mutation of island species. In the long term, understanding the endemic richness and the distribution of island species diversity can develop a conservation plan (Fisher & Robertson 2002; Grytnes & Vetaas 2002).

Langkawi Archipelago (6.3500° N, 99.8000° E) is situated in the north-western part of Peninsula Malaysia and is a part of the Kedah state. Langkawi Archipelago consists of 99 islands (+ 5) small islands visible during low tides) in the Andaman sea. These remote islands' size varies from 0.01–328km^2 and is mainly covered by primary forests (Norhayati et al., 2011). This archipelago covers an area of 47.84km^2. It is approximately 30km from the mainland of Peninsular Malaysia. It is the first global geo-park in Southeast Asia, as it is the only place in Malaysia to have evidence of the complete Palaeolithic era of geological history. The highest peak in Langkawi is the Gunung Raya (881m asl), followed by Gunung Machincang (701m asl), one of the oldest rock formations in Southeast Asia. Langkawi Archipelago has three geo-forest parks, two geological monuments, 97 geo-sites, and nine bio-sites (Komoo, 1999, Norhayati et al., 2011).

In Langkawi Archipelago, surveys were performed by various researchers. Zimmerer (2004), Daicus et al. (2005), Ibrahim et al. (2005), Grismer et al. (2006), Ehwan et al. (2016), and Johana et al. (2016) and had recorded 7, 10, 15, 24, 10 and 23 amphibian species on the main island respectively. While in Pulau Singa Besar, Lim et al. (2010) had documented 14 amphibian species. Presently, 29 amphibians, including a single caecilian species, were reported from this region (Daicus et al., 2005; Lim et al., 2010; Ehwan et al., 2016). However, only limited study areas were explored with a limited time, including many unexplored islands.

As of March 2016, 266 amphibian species had been recorded and described from Malaysia (Inger et al., 2017; Norhayati, 2017). Peninsular Malaysia has 111 species of anurans, including 19 species of bufonids, 20 species of dicroglossids, 12 species of megophryids, 21 species of microhylids, and 18 species ranids, and 21 species of rhacophorids. At the same time, 179 anuran species had been recorded from Sabah and Sarawak (Inger et al., 2017). The exact number of these amphibian species is uncertain as new species are discovered every year (Inger et al., 2017).

There is a high amphibian diversity because of their adaptability towards different environments. Therefore, a high potential for new AMPs to be discovered. Amphibians can inhibit various habitats, from dry, barren desert, saltwater (saline) mangrove areas, primary forests, and polluted urban areas. This environmental variation makes them vulnerable to predators, injuries, infection from pathogens, and even parasites (Clarke, 1997). They counter these threats by their skin secretion, enabling them to exploit a vast habitat and adapt to these environmental conditions. The secretion of frog skin is the primary line of the host's defence system against penetrating infectious pathogens. (Shahabudin et al., 2018; Varga et al., 2019).

Skin secretion from anurans, particularly species from Ranidae families, is a rich source of biologically active peptides (Conlon & Leprince 2009). Anurans' skin secretion can also be used to identify, elucidate evolutionary histories and phylogenetic relationships between species, leading to bioactive compound development with potentials for therapeutic application (Barra & Simmaco, 1995). The pharmaceutical applications of the frog skin secretion protein/peptides include anti-microbial (gram-positive, gram-negative, anti-fungal, anti-MRSA) (Shahabudin et al., 2018; Lekshmipriya et al., 2021), anti-viral (Holthausen et al., 2016; Holthausen et al., 2017; Monteiro et al., 2018), anti-oxidant (Wang et al., 2021), anti-inflammatory (Zhang et al., 2021), anti-diabetic (Conlon et al., 2018), anti-leishmanial (Eaton et al., 2014), anti-biofilm (Grassi et al., 2017), haemolytic (Shahabudin et al., 2018; Jenggut 2019), chemotactic, promotes blood clotting, inhibit the growth of chytrid fungus (frog killing disease) (Holden et al., 2015), helps in wound healing (Sungif et al., 2015), and even anti-cancer properties (Ladram et al., 2016; Conlon et al., 2019; Li et al., 2019).

In recent years, extensive research has been conducted to find bioactive agents, including anti-microbials, effective against these drug-resistant microbes (Shahabudin et al., 2018; Lekshmipriya et al., 2021). These lead to the need to find/discover bioactive peptides as well as AMPs. Bioactive peptides are specific protein fragments that positively affect our body functions and health conditions (Korhonen & Pihlanto, 2006). In contrast, anti-microbial peptides (AMP) refer to the fragments of peptides active against many types of microorganisms such as bacteria, fungus, viruses, parasites, and even a few types of cancerous cells (Sang & Blecha, 2008). Each organism species have different AMPs, including frogs (Hancock & Lehrer, 1998; Hancock & Rozek, 2002; Jenssen et al., 2006)**.**

Globally, AMP of frogs' cutaneous secretions had been grouped from various families, including brevinin-1 and -2, esculentin-1 and -2, ranalexin, ranatuerin-1 and -2, temporin, palustrin, japonicin -1 and -2, tigrinin, nigrocin-2, cancrin-2, guentherin, chclocystokinin, ranakinin, hylaranakinin, brandykinin-like, rugosin, odorranain, and andersonin (Duda et al., 2002; Conlon et al., 2004; Thomas et al., 2012; Kumar et al., 2015). However, only four studies of biomedical AMPs of frog skin secretion were done in Malaysia. That include two investigations from Sarawak (Sabri et al. 2018; Shahabuddin et al., 2018) and the other two in Peninsular Malaysia (Conlon et al., 2008; Dahham et al., 2016). In contrast, only one study had been done from *Polypedates leucomystax* foam nest (Shahrudin et al., 2017).

Generally, AMPs are short molecules that usually consist of 10-100 amino acids, positively charged from +2 to +11. AMPs are positively charged due to the presence of amino acids such as arginine, lysine, histidine**.** They are also amphiphilic and hydrophobic (50% generally) **(**Yeaman & Yount, 2003; Brown & Hancock, 2006; Hancock & Sahl 2006; Niyonsaba et al., 2006; Pasupuleti et al., 2012). Frog AMPs have 9-50 amino acids, positively charged ranging from +1 to +6, and an amphipathic, hydrophobic molecule (Rinaldi et al., 2002; Powers & Hancock 2003; Boland & Separovic 2006; Xiao et al., 2011).

A staggering 10 million mortalities are estimated to occur annually from anti-microbial resistance (AMR) by 2050 (World Health Organization, 2018). The World Health Organisation (WHO) reported that at least 500,000 people had been infected by antibiotic-resistant bacteria across 22 countries (World Health Organization, 2018). These common resistant bacteria include *Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus aureus*, *Streptococcus pneumonia* and *Salmonella spp*. According to the WHO, the AMR-related deadly diseases are pneumonia, tuberculosis, gonorrhoea, and salmonellosis. These diseases are increasing in frequency and are becoming more challenging to treat. AMR occurs naturally in the wild.

Nevertheless, the rate of this resistance has significantly increased due to the misuse of antibiotics. Hence, there is an urgent need for novel drug/ medicine/antibiotic discoveries to combat AMR. AMR's concerns other than human health include animal health, the clinical economy, and the socio-economy (Barriere, 2014; Malik & Bhattacharyya, 2019).

1.2 Problem statement

The problem statements and research gaps of this thesis were:

Island biodiversity shows higher endemism and risk for loss of diversity than the mainland. Besides that, understanding the endemic richness and the distribution of island species diversity can develop a conservation plan. Amphibians are essential for conserving the environment since they act as biological indicators for the environment.

Excluding the main island, Langkawi Archipelago consists of 99 smaller islands. However, the studies on amphibians were previously only done in Langkawi Island, Pulau Tuba, and Pulau Singa Besar. Therefore, amphibians' studies were understudied in this region, especially in smaller islands and sampling sites. Langkawi is the first global geo-park site in Southeast Asia and an important natural heritage site. Tourism based on nature (amphibian) can open a new touristic niche on the island. Amphibians can serve as an essential tourism value since tourism is the primary source of income for the Island.

With the advancing technology, the study on proteomics had been increasing tremendously through recent years. The advancement of proteomics study leads to various benefits in research, such as biomedical, toxicology, phylogenetic, identification of bioactive compounds and others. Frogs are one of the primary sources of bioactive compounds with biomedical and pharmaceutical applications. However, in Malaysia, the studies on proteomics are still in their early stage, especially in identifying and characterising bioactive compounds from natural products for drug discovery purposes.

Anti-microbial resistance (AMR) is a significant threat to human health globally. Diseases caused by AMR are getting more challenging to cure due to the increasing resistance of microbial pathogens. Drugs formulated with anti-microbial peptides (AMP) can be applied to treat these diseases. AMPs are found naturally within organisms, especially amphibians, and is related to the high adaptability of defence mechanisms against various pathogens and predators in the environment. Therefore, there is a need for new drug discovery due to the continuous emergence of drug-resistant microorganisms.

Different frog species have different tolerance to habitat and environmental change. These changes and variations of ecological factors will cause stress to the frog. Frog secretes skin secretion as an effect and adaptation towards these stresses. Unlike genetics, protein expression is influenced by environmental changes. Therefore, there is a need to explore frog species' skin secretion from different habitats to understand the habitat with the protein expressed. Since the protein secreted by frogs of varying environments will be different due to their adaptation.

1.3 Objectives

The objectives of this research are:

1. To evaluate the diversity and distinct habitat assemblages of amphibians in Langkawi Archipelago.
2. To investigate the frogs' skin secretion of the bioactive compound in human commensal and forest edge species.

CHAPTER 2

LITERATURE REVIEW

2.1 Amphibian

2.1.1 Amphibian overview

Amphibians are known as cold-blooded vertebrates. According to Frost (2021), Amphibia's Class contains 8295 species from three Orders, making this class the most diverse vertebrate after fish. The Order Anura means no tail in Latin (An= no, Ura= tail). This order refers to frogs and toads; Order Caudata refers to salamanders and newts; Order Gymnophiona refers to caecilians. These amphibians can be found everywhere globally except in the cold region of the north and south pole. Salamanders and newts possess a long body and a tail. While caecilian, the absence of limbs and is more worm-like than others (Berry, 1975). The ways to identify amphibian species include morphological characteristics, bioacoustics information, and molecular (genetic/protein) analysis.

Their short, stocky body can easily recognise frogs and toads, absence of tail, long muscular hind legs, short forearms, large bulging eyes, and a very wide mouth (Inger et al., 2017). Most frogs possess smooth, slimy skin, while toads have warty rough, dry skin. According to Inger et al. (2017), most amphibians are active during nocturnal, but some species are diurnally active. Frogs and toads undergo metamorphosis. They use bioacoustics to communicate. These calls are separated into several types: mating calls, advertisement calls, response calls, and stress calls (Zainudin et al., 2010; Wilson 2017). Other than bioacoustics, Anuran uses additional physical movements/signalling to communicate. For example, *Staurois guttatus* uses foot-flagging as territorial signals (Grafe & Wanger 2007). In sexual dimorphism, most male individuals were much smaller and more attractive since females need a larger body for reproduction purposes (Woolbright, 1983; Monnet & Cherry, 2002; Liao et al., 2013).

Amphibians are distributed all around the globe. The highest number of amphibian species is in the Neotropical area/South America (2231), followed by Panamanian/southern part of North America (1098) and Oriental/some parts of Asia (1021) (Jenkins et al., 2013; Pimm et al., 2014; AmphibiaWeb, 2020). The high number of amphibian species in the three areas may differ due to the tropical region and climate, which is moist and warm all year round. In comparison, the lowest amphibian species is located in the Saraha-Arabian area (73) since they have the hottest and driest climate. Figure 2.1 indicates the distribution and conservation status of amphibians in the world (AmphibiaWeb, 2020). A higher number of species means there is a higher percentage of loss of species.

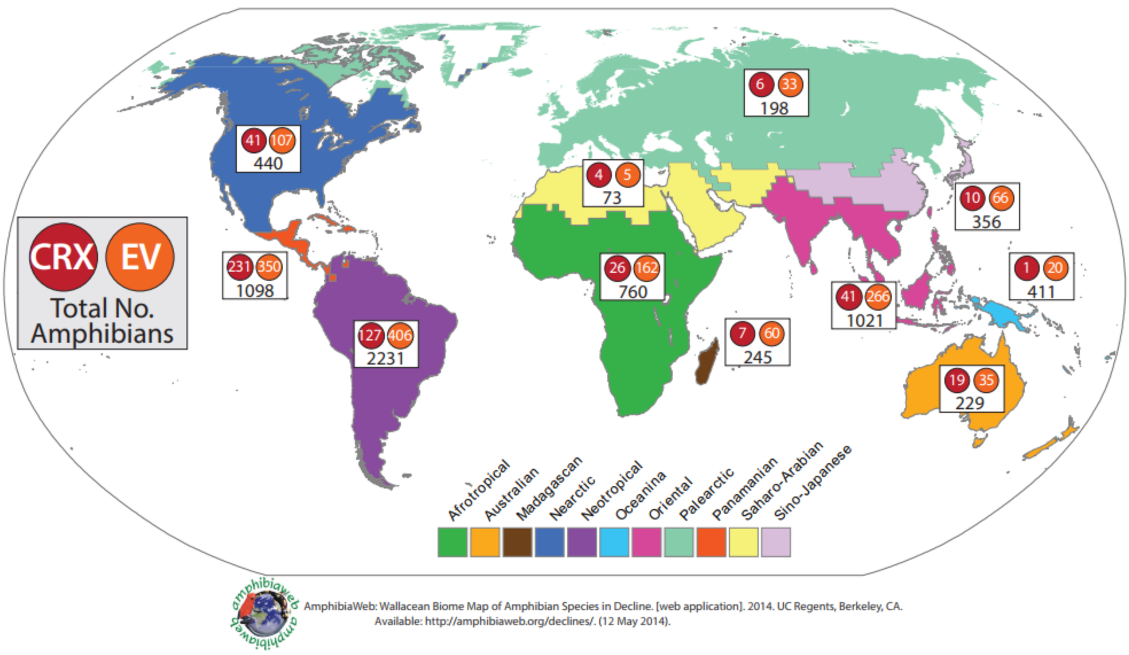

Figure 2.1 Wallacean geographical biome of the distribution and conservation status of amphibians in the world.

Indicator: 1) In red: Number of Extinct, Extinct in the wild or critically endangered species; 2) In orange, Number of Endangered or Vulnerable species; 3) In white, Total number of species within a biome (Amphibiaweb, 2020).

2.1.2 Amphibians of Malaysia

Southeast Asia has a high diversity of amphibians; likewise, many are poorly known and highly threatened species. Two hundred sixty-six amphibian species had been recorded and described from Malaysia as of March 2016 (Inger et al., 2017; Norhayati, 2017). Almost 80 new species had been discovered in the past 20 years. To date (November 2020), 291 amphibian species are recorded in Malaysia (Frost, 2020). The entire species are from seven different families, including Ichthyopiidae (1 genus, 13 species). Bufonidae (9 genera, 49 species), Ceratobatrachidae (1 genus, 2 species), Dicroglossidae (6 genera, 36 species), Megophryidae (3 genera, 40 species), Microhylidae (9 genera, 48 species), Ranidae (13 genera, 14 species) and Rhacophoridae (11 genera, 61 species). According to Norhayati (2017), Peninsular Malaysia has 111 species of anurans, including 19 species of bufonids, 20 species of dicroglossids, 12 species of megophryids, 21 species of microhylids, 18 species of ranids, and 21 species of rhacophorids (Inger et al., 2017; Frost, 2021). Refer to Table 2.1.

Table 2.1. List of family and genus of Malaysian amphibian.

Family	Genus	Species number
Bufonidae	*Ansonia*	23
	Duttaphrynus	1
	Ingerophrynus	7
	Leptophryne	1
	Pelophryne	10
	Phrynoidis	2
	Pseudobufo	1
	Rentapia	2
	Sabahphrynus	1
Ceratobatrachidae	*Alcalus*	2
Dicroglossidae	*Euphlyctis*	1
	Fejervarya	3
	Hoplobatrachus	1
	Limnonectes	26
	Ingerana	1
	Occidozyga	4
Megophryidae	*Leptobrachella*	22
	Leptobrachium	9
	Megophrys	9
Microhylidae	*Chaperina*	1
	Gastrophrynoides	2
	Glyphoglossus	7
	Kalophrynus	18
	Kaloula	3
	Metaphrynella	2
	Microhyla	13
	Micryletta	1
	Phrynella	1
Ranidae	*Abovorana*	2
	Amnirana	1
	Amolops	3
	Chalcorana	3

Family	**Genus**	**Species number**
	Huia	1
	Humerana	1
	Hylarana	2
	Lithobates	1
	Meristogenys	13
	Odorrana	2
	Pulchrana	7
	Staurois	4
	Sylvirana	1
Rhacophoridae	*Chiromantis*	2
	Feihyla	2
	Kurixalus	2
	Leptomantis	10
	Nyctixalus	1
	Philautus	23
	Polypedates	6
	Raorchestes	1
	Rhacophorus	6
	Theloderma	6
	Zhangixalus	2
Ichthyophiidae	*Ichthyophis*	12

In the past, surveys on amphibian diversity were widely done throughout Peninsular Malaysia, especially in the northern part of the country (Perlis, Kedah, Penang, and Perak). In Perlis, a total of 23 species were recorded by Berry (1975), Ibrahim et al. (2001), and Chan et al. (2009). Perak recorded a total of 49 species of amphibians, including two species of caecilian (Grismer et al., 2010; Chan et al., 2010; Ibrahim et al., 2011, 2014; Shahriza & Ibrahim, 2017; Shahrudin & Jaafar, 2014; Hurzaid et al., 2013; Shahriza et al., 2015; Norhayati et al., 2019). Kedah recorded 49 species with two species of caecilian (Daicus et al., 2005; Grismer et al., 2006; Ibrahim et al., 2005; 2006; 2009; 2012; Jaafar et al., 2012; Shahrudin et al., 2011; Shahriza et al., 2011; 2013; 2014; Lim et al., 2010; Shahriza & Ibrahim, 2014; Ehwan et al., 2016). Penang recorded 26 species (Ibrahim et al., 2002; 2008; 2013; Quah et al., 2011).

In Kedah, research on amphibian compositions was conducted various parts of the state, including Gunung Jerai, Gunung Inas, Ulu Paip Recreation Forest, Tupah Recreational Forest, Bukit Perangin, Beris Valley, District IV of MADA Scheme, Bukit Hijau, and Langkawi Archipelago. Fourteen species of amphibians were recorded in Gunung Jerai by Ibrahim et al. (2006); eight species in District IV of MADA Scheme (Ibrahim et al., 2009); 14 species in Beris Valley (Shahrudin et al., 2011); 18 species in Bukit Hijau (Shahriza et al., 2011); 28 species in Gunung Inas (Ibrahim et al., 2012); 15 species in Bukit Perangin (Ibrahim et al., 2012); 13 species in Tupah Recreational Forest (Shahriza et al., 2013); 20 species in Ulu Paip Recreational Forest (Shahriza & Ibrahim, 2014). While in Langkawi Archipelago, a total of 29 species of amphibians were previously recorded by Ng & Ng (1989), Daicus et al. (2005), Ibrahim et al. (2005), Grismer et al. (2006), Norhayati et al. (2007), Lim et al. (2010), Ehwan et al. (2016), and Johana et al. (2016). The results of the previous amphibian surveys in the Langkawi Archipelago were tabulated in Table 2.2.

Table 2.2. Previous surveys on Amphibians of Langkawi Archipelago.

No.	Species name	Ng & Ng (1989)	Daicus et al. (2005)	Ibrahim et al. (2006a)	Grismer et al. (2006)	Norhayati et al. (2007)	Lim et al. (2010)	Ehwan et al. (2016)	Johana et al. (2016)	Lim et al. (2010)-compiled list
		Pulau Langkawi	Lubuk Semilang and Durian Perangin	Pulau Langkawi	Pulau Langkawi	Pulau Tuba	Pulau Singa Besar	Gunung Raya	Pulau Langkawi	Langkawi Archipelago
	GYMNOPHIONA									
	Ichthyophiidae									
1.	*Ichthyophis sp*	**x**	**-**	**-**	**x**	**-**	**x**	**x**	**x**	**x**
	ANURA									
	Bufonidae									
2.	*Duttaphrynus melanostictus*	**-**	**x**	**x**	**x**	**x**	**x**	**-**	**x**	**x**
3.	*Ingerophrynus parvus*	**-**	**-**	**-**	**-**	**-**	**x**	**x**	**x**	**-**
4.	*Phrynoidis asper*	**-**	**-**	**x**	**x**	**-**	**x**	**x**	**x**	**x**

No.	Species name	Ng & Ng (1989)	Daicus et al. (2005)	Ibrahim et al. (2006a)	Grismer et al. (2006)	Norhayati et al. (2007)	Lim et al. (2010)	Ehwan et al. (2016)	Johana et al. (2016)	Lim et al. (2010)-compiled list
		Pulau Langkawi	Lubuk Semilang and Durian Perangin	Pulau Langkawi	Pulau Langkawi	Pulau Tuba	Pulau Singa Besar	Gunung Raya	Pulau Langkawi	Langkawi Archipelago
	Dicroglossidae									
5.	*Fejervarya cancrivora*	**-**	**-**	**x**	**x**	**-**	**x**	**-**	**x**	**x**
6.	*Fejervarya limnocharis*	**x**	**x**	**x**	**x**	**x**	**-**	**x**	**x**	**x**
7.	*Limnonectes blythii*	**-**	**x**	**x**	**x**	**-**	**x**	**-**	**x**	**x**
8.	*Limnonectes malesianus*	**-**	**x**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
9.	*Limnonectes hascheanus*	**-**	**-**	**-**	**x**	**-**	**x**	**x**	**x**	**x**
10.	*Limnonectes macrognathus*	**-**	**-**	**-**	**x**	**-**	**x**	**x**	**x**	**x**

No.	Species name	Ng & Ng (1989)	Daicus et al. (2005)	Ibrahim et al. (2006a)	Grismer et al. (2006)	Norhayati et al. (2007)	Lim et al. (2010)	Ehwan et al. (2016)	Johana et al. (2016)	Lim et al. (2010)-compiled list
		Pulau Langkawi	Lubuk Semilang and Durian Perangin	Pulau Langkawi	Pulau Langkawi	Pulau Tuba	Pulau Singa Besar	Gunung Raya	Pulau Langkawi	Langkawi Archipelago
11.	*Occidozyga sumatrana*	**-**	**-**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
12.	*Occidozyga lima*	**-**	**-**	**x**	**x**	**-**	**-**	**-**	**x**	**x**
13.	*Occidozyga martensii*	**-**	**-**	**-**	**x**	**-**	**-**	**-**	**x**	**x**
	Megophryidae									
14.	*Leptobrachium smithi*	**-**	**-**	**x**	**x**	**-**	**x**	**x**	**x**	**x**
15.	*Megophrys aceras*	**-**	**-**	**-**	**x**	**-**	**-**	**x**	**x**	**x**
	Microhylidae									
16.	*Kaloula pulchra*	**-**	**-**	**x**	**x**	**x**	**x**	**-**	**x**	**x**

No.	Species name	Ng & Ng (1989)	Daicus et al. (2005)	Ibrahim et al. (2006a)	Grismer et al. (2006)	Norhayati et al. (2007)	Lim et al. (2010)	Ehwan et al. (2016)	Johana et al. (2016)	Lim et al. (2010)-compiled list
		Pulau Langkawi	Lubuk Semilang and Durian Perangin	Pulau Langkawi	Pulau Langkawi	Pulau Tuba	Pulau Singa Besar	Gunung Raya	Pulau Langkawi	Langkawi Archipelago
17.	*Microhyla berdmorei*	-	x	-	x	-	-	-	x	x
18.	*Microhyla butleri*	-	-	x	x	-	-	-	x	x
19.	*Microhyla fissipes*	-	-	-	x	-	-	-	x	x
20.	*Microhyla heymonsi*	-	-	x	x	-	-	-	x	x
21.	**Rhacophoridae**									
	Raorchestes parvulus	-	-	-	x	-	-	-	x	x
22.	*Polypedates leucomystax*	-	x	x	x	-	x	-	x	x
23.	*Rhacophorus bipunctatus*	-	-	-	x	-	-	-	-	x

No.	Species name	Ng & Ng (1989) Pulau Langkawi	Daicus et al. (2005) Lubuk Semilang and Durian Perangin	Ibrahim et al. (2006a) Pulau Langkawi	Grismer et al. (2006) Pulau Langkawi	Norhayati et al. (2007) Pulau Tuba	Lim et al. (2010) Pulau Singa Besar	Ehwan et al. (2016) Gunung Raya	Johana et al. (2016) Pulau Langkawi	Lim et al. (2010)-compiled list Langkawi Archipelago
24.	*Polypedates discantus*	**-**	**-**	**-**	**-**	**-**	**-**	**x**	**x**	**-**
	Ranidae									
25.	*Amolops larutensis*	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**x**
26.	*Hylarana erythraea*	**x**	**x**	**x**	**x**	**x**	**x**	**-**	**x**	**x**
27.	*Pulchrana glandulosa*	**-**	**-**	**x**	**x**	**-**	**-**	**-**	**x**	**x**
28.	*Chalcorana labialis*	**-**	**x**	**x**	**x**	**-**	**x**	**-**	**-**	**x**
29.	*Odorrana hosii*	**-**	**x**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
	Total species	**3**	**9**	**15**	**24**	**5**	**14**	**10**	**23**	**26**

Note: "**x**" = present, "**-**"= absent

2.1.3 History of amphibian studies in Langkawi Archipelago

Studies of amphibians in the Malay Peninsula started in the 20th century. One of the earliest records of amphibians in the Malay Peninsula was reported by Smith (1930) in his book entitled 'Reptilia and Amphibia of the Malay Peninsula.' He recorded the finding of *Limnonectes macrognathus* and *Limnonectes hascheanus* in the extreme northern part of the Malay Peninsula (Langkawi Archipelago). His records also include a detailed morphological description for both species. For *Limnonectes macrognathus*, the morphological of sexual dimorphism between males and females and the description of tadpoles were pointed out. While for *Limnonectes. hascheanus*, the description of adult males and eggs were specified.

In 1975, Berry had published a book entitled 'The Amphibian Fauna of Peninsular Malaysia.' In the book, he had mentioned eight species of frogs that inhabit Langkawi island. These including *Fejervarya cancrivora* (*Rana cancrivora*), *Fejervarya limnocharis* (*Rana limnocharis*), *Limnonectes blythii* (*Rana macrodon*), *Occidozyga sumatrana* (*Occidozyga laevis*), *Microhyla berdmorei*, *Microhyla fissipes* (*Microhyla ornata*), and *Hylarana erythraea* (*Rana erythraea*). He also describes morphological description, colour in life, ecological notes, and known locality.

The article published by Ng & Ng (1989) recorded caecilian species (Ichthyophis sp.) and two frog species, *Hylarana erythraea* and *Fejervarya limnocharis* in Langkawi freshwaters. Zimmerer (2004) had noted seven species of frogs in his book. These include two species of megophryrids, two species of bufonids, a single species of microhylid, a single species of ranid, and a single species of dicroglossid. Daicus et al. (2005) recorded nine amphibian species in Lubuk Semilang and Durian Perangin

Recreational Park. The species consist of two bufonids, three dicroglossids, three ranids, a single microhylid species, and a single rhacophorid species. However, they only surveyed a small part of the island within a limited timeframe.

Extensive research of amphibians throughout the island was only done in the early 21st century by Ibrahim et al. (2005) and Grismer et al. (2006). They recorded 15 and 24 species of amphibians, respectively. Ibrahim et al. (2005) had recorded two species of bufonids, five species of dicroglossids, one species of megophyrids, three species of microhylids, three species of ranids, and a single species of rhacophorid. At the same time, Grismer et al. (2006) recorded a caecilian species, two species of bufonids, eight species of dicroglossids, two species of megophyrids, five species of microhylids, three species of ranids, and three species of rhacophorid.

Grismer et al. (2006) had done a compilation and commentary on each amphibian species. A preliminary account of amphibians in Pulau Tuba was done by Norhayati et al. (2007). She recorded five amphibian species including, *Duttaphrynus melanostictus*, *Fejervarya limnocharis*, *Occidozyga sumatrana* (*Occidozyga laevis*), *Kaloula pulchra*, and *Hylarana erythraea*.

Since 2010, a new era of research on amphibians of Langkawi Archipelago was done. More effort was invested with a focus on a specific area at a time. For example, Lim et al. (2010) had recorded 14 amphibian species from Pulau Singa Besar within 32 days, from three different months. They documented a single caecilian species (Ichthyophis sp.), three bufonids, five dicroglossids, one megophryid, one rhacophorid, and two ranids.

Ten amphibian species, including a caecilian, were recorded from Gunung Raya via a passive sampling method (Ehwan et al., 2016). These species included two bufonids, one rhacophorid, two megophyrids, and four dicroglossids. Moreover, the evaluation of species composition, diversity, and relative abundance of amphibians on Langkawi Island was done by Johana et al. (2016). She had recorded 23 species of amphibians.

Lim et al. (2010) and Norhayati et al. (2011) compiled the amphibian species that inhabit the Langkawi Archipelago. They reported 25 and 26 species of amphibians, respectively. From the compilation, 26 amphibians on Langkawi Island, five species on Pulau Tuba and 14 from Pulau Singa Besar.

2.1.4 Amphibian threats

Habitat loss is the most critical cause of the decline and extinction of amphibian species (Hamer 2016; Mayani-Parás et al., 2019). Many amphibian species are vulnerable to habitat loss, mainly losing their breeding ground/habitat (Almeida-Gomes & Rocha, 2015). Habitat fragmentation refers to the separate patches of the forest from the continuous forest and eventually separated from one another, causing changes in habitat quality that may indirectly affect amphibian populations (Delaney et al., 2021). Habitat alterations include forest land conversion into agriculture, deforestation, urbanisation, and human activities (Sodhi et al., 2010a, 2010b).

The infectious disease that causes a decline in amphibians' population includes chytridiomycosis (fungus), Ranavirus disease (virus), saprolegniosis (water mould), and *Ribeiroia sp.* (trematode parasite) (Carey, 1993; Berger et al., 1998; Daszak et al., 1999; Johnson et al., 1999; Kiesecker et al., 2001; Daszak et al., 2003). Chytridiomycosis causes sporadic death in some amphibian populations and 100% mortality in others. It is caused by *Batrachochytrium dendrobatidis* (Bd fungus), commonly known as Chytrid fungus (Daszak et al., 2003). This fungus infects the surface layers, causing damage to the frog's skin's outer keratin layer, disrupting the skin's normal functions, such as respiration and moisture control (Daszak et al., 1999)**.**

Amphibians spend most of their lifetime in the aquatic environment; therefore, they are susceptible to contaminations and environmental changes. Several pollutions cause and affect the decline of amphibians' population, such as water pollution, especially from pesticides, soil pollution, light pollution, and even noise pollution (Verrell, 2000; Blaustein et al., 2003; Tennessen et al., 2014; May et al., 2019). The sensitivity of amphibians towards the environment is because amphibians' skins are susceptible to water penetration, gas, and even ion transport from the ground (Verrell, 2000). Therefore, they are important indicators of the environment, especially water quality. Light pollution changes light conditions due to human activities; it reduces egg hatching success, and tadpoles tend to be less active than usual (May et al., 2019). Simultaneously, anthropogenic noise pollution causes physiological stress and alters frogs' breeding behaviour (Tennessen et al., 2014).

Waterbody habitat is the most crucial aspect of amphibians' life cycle, especially during their larval/tadpole and reproductive stage. This type of habitat serves as their breeding and larval-developmental site. Waterbody of shallow ponds, seasonal pools, wetlands, and even slow backwater off-channel streams, was preferred due to the absence of a fish predator, presence of fewer invertebrates. Furthermore, these habitats have warmer waters suitable for egg and larval development (Barnett, 2005). Some species prefer to live closer to the water line zone to reduce/avoid predators during their daily activities such as foraging, survival, and ecological function around the riparian zone (Heard et al., 2008; Halliday, 2008).

Among vertebrates, the amphibian is the only group without physical protection on their skin, such as furs on mammals, feathers on birds, scales on fishes, and reptiles. That makes their skin to be more vulnerable to the environment than other animals. These vulnerabilities include the loss of moisture from their body and the pathogenicity of pathogens (Clark, 1997). Therefore, the skin of amphibians is vital for their survival. Amphibian skin also acts as a respiratory mechanism of amphibians in water via dissolved oxygen absorbance through their skin. They retain moisture towards the individual, drinking (osmoregulation) via water absorbance directly into their skin and even as a protective layer via secretions, especially in their immune system. (Bentley et al., 1979; Suzuki et al., 2007; Ogushi et al., 2010).

2.2 Frog secretions as a defence mechanism

2.2.1 Skin secretions

The primary function of the skin secretion in the frog acts as its protective defence mechanism. This includes avoiding predation since it makes the body of the individual slippery and enables the individual to escape from predators/danger (Stebbins & Cohen, 1997). Some of the species possess secretions that contain natural 'super glue' like substances, which can be used to immobilise a predator (Evans & Brodie, 1994). These secretions contain various anti-microbial/defence peptides (AMPs) against pathogens in the environment (Varga et al., 2019). The secretions make the amphibians unpalatable and toxic to their predator (Santos et al., 2016; Schulte et al., 2017).

The glandular tissue secreted the skin secretions of frogs, usually situated at the individual's dorsal/lateral side. All adult frogs possess two dermal glands: glandular and mucous glands (Duellman & Trueb, 1994; Toledo & Jared, 1995). refer to Fig 2.2. These skin glands also produce proteins and peptides such as neuropeptides, immunoglobulins, skin peptides and lysozymes (Rollins-Smith & Conlon, 2005; Ramsey et al., 2010; Rollins-Smith et al., 2011). These secretions will be secreted onto the skin surface after the individual was induced by stress. This stress will trigger the release of epinephrine and norepinephrine hormones. These hormones were released by