INTEGRATIVE TAXONOMY OF THE *LIMNONECTES HASCHEANUS-LIMBORGI* (ANURA: DICROGLOSSIDAE) COMPLEX: RESOLVING CRYPTIC SPECIES IN PENINSULAR MALAYSIA

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by

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LIST OF ABBREVIATIONS

1stTL	Length of 1st Toe
AAH	Amirrudin Ahmad Herpetofauna collection, Universiti Malaysia Terengganu
aLRT	Approximate Likelihood Ratio Test
BI	Bayesian Inference
BIC	Bayesian Information Criterion
CA	Cloaca
CAS	China Academy of Sciences
CV	Coefficient of Variation
DA	Discriminant Function Analysis
DAPC	Discriminant Analysis of Principal Components
ddH_2O	Double-distilled Water
EL	Eye Diameter
EN	Front of Eyes to the Nostril
ESHQ	Evan S.H. Quah field series
ESS	Effective Sample Size
F	Female
FAL	Forearm Length
FFT	Fast Fourier Transform
FH	Fraser's Hill
FMNH	Field Museum of Natural History
FOL	Foot Length
GT	Genting Highlands
HAL	Hand Length
HL	Head Length
HML	Herpetological Museum Laboratory
HPD	Highest Posterior Density
HW	Head Width
IMTL	Length of Inner Metatarsal Tubercle
IN	Internarial Distances
IOD	Interorbital Distances
KD	Kedah

KUHE	Graduate School of Human and Environment Studies, Kyoto University
LAL	Distance from the axilla to flexed elbow
LSUHC	La Sierra University Herpetological Collection
M	Male
MBE	Mandible to Back of the Eye
MCMC	Markov Chain Monte Carlo chains
MFE	Mandible to Front of the Eye
ML	Maximum Likelihood
MN	Mandible to Nostril
mtDNA 16S rRNA	Mitochondrial 16S ribosomal RNA
MVZ	Museum of Vertebrate Zoology
Mya	Million Years Ago
Na_2EDTA	Disodium Ethylenediaminetetraacetic Acid
NaCl	Sodium Chloride
NS	Nostril-Snout Length
nuDNA Tyr	Nuclear DNA Tyrosine
PCA	Principal Component
PCR	Polymerase Chain Reaction.
PEN	Penang Island
PERMANOVA	Permutational Multivariate Analysis of Variance
pH	Potential of Hydrogen
PH	Pahang
PITC	Perak Integrated Timber Complex
PK	Perak
PL	Perlis
PM	Peninsular Malaysia
SAP	Sekayu Agricultural Park
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SH-aLRT	Shimodaira-Hasegawa approximate Likelihood Ratio Test
SL	Snout Length
SPA	Supra-Acillary
SVL	Snout-Vent Length
SWFU	Southwest Forestry University

TD	Tympanum Diameters
TFOL	Length of Tarsus and Foot
THIGL	Thigh Length
THNHM	Thailand Natural History Museum
TL	Tibia Length
TR	Terengganu
Tris-HCL	Tris Hydrochloride
UEW	Width of Upper Eyelid
UFBS	Ultrafast Bootstrap
USMHC	Universiti Sains Malaysia Herpetological Collection
UV	Ultraviolet
X	Mean

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TAKSONOMI INTEGRATIF KOMPLEKS *LIMNONECTES HASCHEANUS-LIMBORGI* (ANURA: DICROGLOSSIDAE): MENYELESAIKAN SPESIES KRIPTIK DI SEMENANJUNG MALAYSIA

ABSTRAK

Limnonectes hascheanus dan *Limnonectes limborgi*, yang berkait rapat dan dirujuk sebagai kompleks *Limnonectes hascheanus-limborgi*, menunjukkan perbezaan evolusi yang jelas di Semenanjung Malaysia. Sebelum ini, hanya *L. hascheanus* yang dilaporkan terdapat di rantau ini. Namun, Matsui mengenal pasti satu spesimen dari Janda Baik sebagai spesies *L. limborgi*. Kajian ini bertujuan untuk menjelaskan status kedua–dua spesies di Semenanjung Malaysia melalui pendekatan bersepadu, yang melibatkan data morfologi, bioakustik, dan molekul. Hasil kajian menunjukkan terdapat dua leluhur berbeza dalam kompleks *Limnonectes hascheanus-limborgi* yang dapat dibezakan berdasarkan genetik, panggilan iklan jantan, dan morfologi luaran. Kedua-dua leluhur ini menunjukkan perbezaan genetik yang signifikan, dengan jarak 3.4–7.8% berdasarkan urutan gen mtDNA 16S rRNA dan nuDNA Tyrosinase. Leluhur pertama, dikenali sebagai morfotip *L. hascheanus*, sejajar dengan ciri-ciri spesimen dari Pulau Pinang, termasuk badan yang langsing, warna perang zaitun terang atau gelap, dan selaput kaki yang tidak mencapai tuberkel subartikular tengah pada jari keempat. Spesimen jantan tidak mempunyai odontoid yang membesar. Populasi dari Pulau Langkawi, Kedah, Perlis, dan beberapa dari Bukit Larut, Perak, menunjukkan persamaan genetik dan bioakustik dengan spesimen topotip *L. hascheanus*. Morfotip kedua, yang menyerupai *L. limborgi* dari Myanmar, mempunyai SVL yang lebih besar, selaput penuh yang mencapai tuberkel subartikular tengah pada jari keempat, dan odontoid yang membesar pada spesimen jantan. Populasi dari Bukit Fraser, Genting

Highlands, Pulau Tioman, Terengganu, dan beberapa spesimen dari Bukit Larut adalah lebih dekat secara genetik dengan bentuk ini, yang menunjukkan kemungkinan sinonim. Analisis bioakustik turut membezakan garis keturunan ini: Morfotip *L. hascheanus* menghasilkan panggilan yang terdiri daripada 21–34 nota dalam pola panggilan tunggal tanpa modulasi, manakala panggilan dari populasi Bukit Fraser dan Genting Highlands termasuk dua jenis nota dan biasanya terdiri daripada 1–6 nota bagi setiap panggilan. Perbezaan turut diperhatikan pada tempoh panggilan, kadar nota, selang antara nota, tempoh nota, bilangan denyut, dan frekuensi dominan. Kedua-dua jenis panggilan telah direkodkan daripada jantan simpatrik yang memanggil sebelah menyebelah di Bukit Larut. Akhir sekali, variasi genetik antara populasi *L. limborgi* dari Semenanjung Malaysia, Myanmar, dan kawasan lain mencadangkan kemungkinan wujudnya spesies kriptik dalam *L. limborgi*.

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ABSTRACT

Limnonectes hascheanus and *L. limborgi* are two closely related species that are herein referred to as the *Limnonectes hascheanus–limborgi* complex. In the past, *L. hascheanus* was the only species reported to be present in Peninsular Malaysia. Matsui reported one of their examined specimens from Janda Baik as *L. limborgi*. This study aimed to ascertain the status of both species in Peninsular Malaysia through an integrated approach, combining morphological, bioacoustic and molecular data. The results indicated that in Peninsular Malaysia there two different lineages of the *L. hascheanus–limborgi* complex that can be separated genetically, by their male advertisement calls, as well as by external morphology. Both lineages differ by having a high genetic distance between them of 2.4–7.8% based on both mtDNA 16S rRNA and nuDNA Tyrosinase genes. One lineage has a morphotype assignable to *L. hascheanus* described from Penang Island and bears the following characteristics: slender body and light or dark–olive–brown color; toe webbing not reaching middle subarticular tubercle on the fourth toe; and the absence of enlarged odontoids in males. Populations from Langkawi Island; Kedah, Perlis, and some specimens from Bukit Larut, Perak are genetically and bioacoustically similar to topotypic specimens of *L. hascheanus* from Penang Island. The second morphotype differs from the former by having a larger SVL; full webbing reaching middle subarticular tubercle on the fourth

toe; and the presence of enlarged odontoids in males. It is morphologically most similar to *L. limborgi* from Myanmar. Populations from Fraser's Hill, Genting Highlands, Tioman Island, Terengganu and some specimens from Bukit Larut are genetically closer to this latter form and thus considered synonymous. Analyses of the calls also revealed differences in both lineages with *L. hascheanus* having a call pattern with no offset of a single note and consisted of 21–34 notes as one call. Whereas recordings of the populations from Fraser's Hill and Genting Highlands were composed of two types of notes and calls and usually consist of 1–6 notes. Other differences can also be observed from call duration, note rate, inter–note interval, note duration, note period, pulses per call and dominate frequency. Both call types were heard by syntopic males calling side by side at Bukit Larut. Variation was observed between the populations of *L. limborgi* from Peninsular Malaysia and Myanmar, as well as populations from other countries. This suggests the possibility of cryptic species within *L. limborgi*.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Amid large–scale habitat destruction and a rising number of threatened species in the world's biodiversity hotspots such as the Amazon rainforest, Madagascar, and Southeast Asia, taxonomic research in these regions is being conducted with increasing urgency (Brooks *et al*., 2002; Mittermeier *et al*., 2011; Luedtke *et al.,* 2023). Currently, amphibians are considered among the most threatened animals globally due to their ecological sensitivity, extensive habitat loss, and the global spread of fatal diseases (Collins & Storfer, 2003; Mendelson *et al*., 2006; McLeod, 2010; Catenazzi, 2015; Luedtke *et al*., 2023). Therefore, taxonomic research on amphibians in global biodiversity hotspots is a high priority.

Due to its ancient tropical rainforest, Peninsular Malaysia, is considered a biodiversity hotspot and hosts a wide variety of environments that support a remarkable diversity of amphibians. According to the latest checklist by Chan *et al*., (2010), 107 species were documented in the region. Subsequent updates, as reported by Frost (2024), have increased this number to 115 species (Grismer, 2011; Chan *et al*., 2014; Hasan *et al*., 2014; Matsui *et al*., 2017; Chan *et al*., 2018; Sheridan & Stuart 2018; Chan *et al*., 2020). Many of these newly discovered species have emerged from the division of previously identified single widespread species into multiple distinct species. This phenomenon highlights the challenge of identifying and categorizing species within morphologically cryptic groups, where a significant proportion of species may remain undiscovered.

Limnonectes hascheanus and *L. limborgi* are two very similar-looking and closely related species. They are sometimes referred to as the *Limnonectes*

hascheanus–limborgi complex (Inger & Stuart, 2010). Inger & Stuart (2010) tackled the systematics of the complex and confirmed *L. limborgi's* status as a distinct species and not a junior synonym to *L. hascheanus* by providing molecular data and diagnostic morphological characters. The geographic ranges of the two species were also reported to be distinct with *L. limborgi* being distributed from southern Myanmar northward towards northern Thailand and Laos before curving around central Laos, northeastern Thailand, Cambodia, and southern Vietnam. Whereas *L. hascheanus* is mainly restricted to the southern part of the Thai-Malay Peninsula and scarcely overlapping with *L. limborgi* at Kaeng Krachan, Thailand (Inger & Stuart, 2010).

Based on the delimitation of their distributions by Inger & Stuart (2010), the species that occurs in Peninsular Malaysia should only be *L. hascheanus*. However, Matsui *et al*., (2014a) demonstrated that a specimen of *L. limborgi* was obtained from Janda Baik, Peninsular Malaysia. In addition, personal encounters by L. Lee. Grismer & Evan Quah (pers. comm.) suggest that *L. limborgi* is more widespread on Peninsular Malaysia and it occurs synoptically with *L. hascheanus* in some areas of the country. The evidence suggests that reports of the occurrence of *L. hascheanus* in some parts of Peninsular Malaysia in the literature might be erroneous identifications of *L. limborgi*. Hence, there is a need to re-examine all available material of the two species in Peninsular Malaysia using genetic data and detailed morphological analyses to correctly identify each species and ascertain the accurate distribution of their populations.

Besides that, the two species lack bioacoustics comparison which is a very useful as a method to differentiate anuran species-complexes in the field. This method has been successfully applied to distinguish new species such as *Limnonectes bagoyoma* and *L. bagoensis* from Myanmar, *Pulchrana sundabarat* and *P. banjarana*

from Peninsular Malaysia (Chan *et al*., 2020b; Köhler *et al.*, 2021). Therefore, bioacoustics analysis of the calls of *L. hascheanus* and *L. limborgi* need to be evaluated to aid identification and compliment the findings of Inger & Stuart (2010) that they are distinct species.

1.2 Hypothesis

1.2.1 Null hypothesis: The *L. hascheanus–limborgi* species complex in Peninsular Malaysia comprises a single species, with no significant genetic, morphometric, or bioacoustic differences among its populations.

1.2.2 Alternative Hypothesis: The *L. hascheanus–limborgi* species complex is composed of more than one species in Peninsular Malaysia. The species of this complex should be distinguishable genetically, morphometrically, and bioacoustically.

1.3 Objectives

1.3.1 Objective 1

To reexamine the systematics of the *L. hascheanus–limborgi* species complex in Peninsular Malaysia using genetic data.

1.3.2 Objective 2

To reexamine the systematics of the *L. hascheanus–limborgi* species complex in Peninsular Malaysia using additional morphological characters for more accurate identification and differentiation.

1.3.3 Objective 3

To revise the current distribution of the *L. hascheanus–limborgi* species complex in Peninsular Malaysia.

1.3.4 Objective 4

To describe and compare the advertisement calls of the populations within the *L. hascheanus–limborgi* species complex populations from Peninsular Malaysia.

1.4 Research Flow Chart

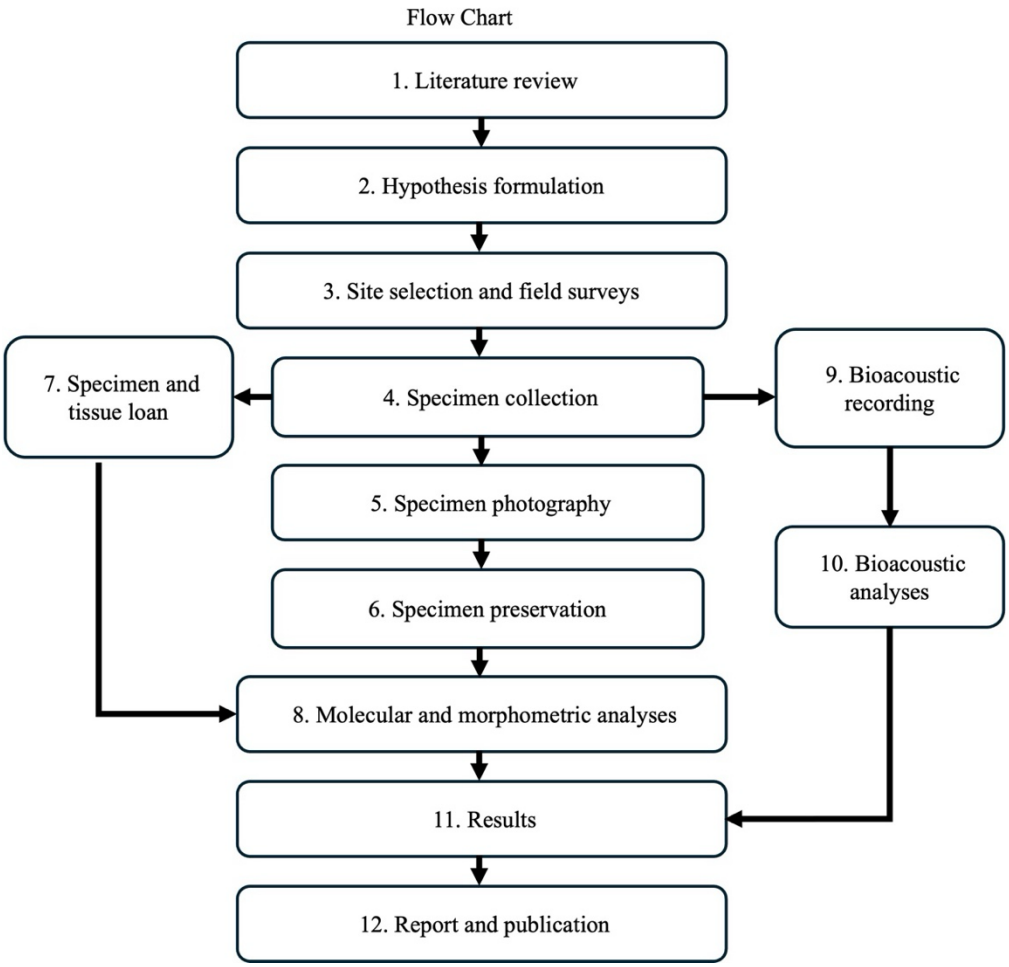

Figure 1.1 Flow chart of study.

CHAPTER 2

LITERATURE REVIEW

2.1 Ecology of amphibians

Amphibians are ectothermic, tetrapods that inhabit both water and land. The term 'amphibian' originates from the Greek words “amphi” and “bio” meaning 'both kinds of life' and was initially used to describe animals capable of living in both land and water. Biologists have classified amphibians into three major groups: salamanders (Caudata), caecilians (Gymnophiona), and frogs and toads (Anura) (Dodd, 2010). Among these groups, the order Anura (frogs and toads) is the largest, with around 8,588 species known worldwide (Frost, 2024). Anurans are distinguished by their unique features compared to other tetrapods. They have broad, flat heads and longer hind limbs, which are adapted for jumping, swimming, walking, running, and climbing. These distinct characteristics make them easily recognizable (Pough, 2007). Anurans inhabit almost every environment, including water, land, underground, and trees, even in areas with low temperatures and extremely dry conditions (Herreid & Kinney, 1967; Predavec & Dickman 1993; Larson *et al*., 2014). Furthermore, the reproductive behavior of anurans often involves vocalization, with males emitting calls to attract potential mates during the mating season. This complex interplay of physiological adaptations and behavioral strategies underscores the evolutionary success of anurans across various ecosystems (Cocroft & Ryan, 1995; Tobias *et al*., 2011; Gilbert & Bell, 2018).

2.2 Current state of amphibians worldwide

Since 1985, there has been a significant increase of over 60% in the total number of amphibian species described (AmphibiaWeb, 2023). To date,

approximately 8,588 species are recognized worldwide (Frost, 2024), and this number is expected to continue to increase, with more than 100 new species being described every year since 2004 (AmphibaWeb, 2018). Amphibians exhibit one of the highest proportional rates of new species discovery among all vertebrate groups (Hanken, 1999). Despite these ongoing discoveries, it is globally recognized that amphibians are still in decline, and the situation may only be worsening (Stuart *et al*., 2004). According to the IUCN (2022), amphibians rank as the most threatened vertebrate group, as indicated by the Red List Index (RLI), with their decline occurring at a more accelerated pace compared to birds and mammals. Furthermore, based on the IUCN Red List report (Version 2022-2), approximately 41% of all recognized amphibian species, with estimates ranging from 30% to 50%, are classified as threatened. This classification includes species listed as Critically Endangered (CR), Endangered (EN), and Vulnerable (VU). Among anurans, 46% (3027 species) are categorized as 'low risk/least concern,' while 15.2% (1000 species) are labelled as 'data deficient' (IUCN, 2022).

The decline of amphibian populations can be attributed to several factors, including habitat destruction, pollution, climate change, and competition and predation by invasive species, as well as various anthropogenic influences (Collins & Storfer, 2003; Mendelson *et al*., 2006; McLeod, 2010). Amphibians, with their acute sensitivity to changes in both terrestrial and aquatic microhabitats, often serve as vital environmental indicators for assessing habitat health and tracking climate change. Recognizing the urgency of this issue, research efforts are channelled towards prioritizing conservation strategies aimed at protecting global amphibian populations and enhancing the effectiveness of conservation endeavours (McLeod, 2010).

2.3 Amphibians of Peninsular Malaysia

With its complex geological history and climatically diverse conditions, Peninsular Malaysia has long been known to harbour a high degree of herpetological endemicity, including amphibians (Ibrahim *et al*., 2011; Sumarli *et al*., 2015; Chan *et al*., 2019). The study of amphibians in Peninsular Malaysia can be traced back to the late 19th and early 20th centuries, with early explorers making notable contributions (Chan & Ahmad *et al*., 2021a). During that period, Butler (1902) and Robinson (1905) created the first checklist that recorded 63 species of amphibians. As research continued, the checklist was constantly being updated, and the number of species continued to rise (Chan *et al*., 2010).

The latest checklist published by Chan *et al*., (2010) documented 107 species of amphibians (105 anurans; 3 caecilians) in Peninsular Malaysia. This number has continued to rise, with numerous new species discovered since 2010. However, many of these new discoveries have resulted from splitting previously recognized single widespread species into multiple species. For example, *Polypedates discantus* by Rujirawan *et al*., (2013), *Sylvirana malayana* by Sheridan & Stuart (2018), *Abavorana nazgul* by Quah *et al*., (2017), and *Amolops gerutu* and *Amolops australis* by Chan *et al*., (2018).

Using the example of *S. malayana*, before 2018, it was recognised as *Sylvirana nigrovittata* that was considered a single species with a broad geographic range in mainland Southeast Asia, extending from northeastern India to Peninsular Malaysia (Sheridan & Stuart, 2018). Following taxonomic revision by Sheridan & Stuart (2018), the geographic range of *S. nigrovittata* was redefined, and five new species were identified. The species previously grouped under *S. nigrovittata* in Peninsular Malaysia are now recognized as a distinct species known as *S. malayana*, and *S.*

nigrovittata is no longer considered part of the Malaysian anuran assemblage (Sheridan & Stuart 2018). Furthermore, Sheridan & Stuart (2018) suggested that three of the five newly described species (*S. lacrima, S. roberti,* and *S. annamitica)* may be threatened due to habitat loss and their limited geographic distributions. This underscores the importance of taxonomic studies as a fundamental basis for biodiversity conservation efforts.

2.4 Taxonomic challenges of cryptic species

Cryptic species are genetically distinct but morphologically similar species that are currently (or historically) classified as a single species. The identification and description of cryptic species pose challenges that hinder conservation efforts (Bickford *et al*., 2007), especially for widely distributed species complexes that might be incorrectly classified as "least concern" or "known not to be threatened" due to the presence of cryptic evolutionary lineages (McLeod, 2010; Suwannapoom *et al*., 2012; Herr *et al*., 2021). These cryptic lineages may consist of multiple independently evolving species that have not yet been described or exhibit minimal observable phenotypic differences. Consequently, cryptic species are often misidentified during field surveys, leading to an underestimation of biodiversity and inadequate conservation measures for these unrecognized species.

The concept of cryptic species presents a paradox, as many studies that claim to reveal them often rely on traditional morphological diagnostics, using historical traits specific to certain taxa to distinguish species within the same groups (Grismer *et al*., 2018). This reliance on morphology frequently conflicts with molecular evidence, raising questions about the true nature of cryptic species. While molecular techniques have improved the identification of species that are genetically distinct but

morphologically similar, retrospective morphological analyses can still provide independent diagnoses (Wood *et al*., 2009; Grismer *et al*., 2013a, b, 2018). This contradiction underscores the challenges of defining and identifying cryptic species, suggesting that a combined approach using both morphological and molecular data is essential for accurate species delimitation.

Given these complexities, it is not surprising that advancements in DNA sequencing and phylogenetic analysis have led to the development of various molecular-based species delimitation methods. Molecular techniques have greatly enhanced taxonomy by standardizing biodiversity analysis, formalizing species delimitation methods, increasing transparency, and accelerating the identification of new species (Lukhtanov, 2019). These methods assist researchers in tackling the difficulties of identifying hidden or poorly understood cryptic lineages and facilitate a more detailed examination of species-level differences.

Among anurans, new species are discovered annually, with DNA barcoding serving as a valuable tool for rapidly identifying potential undescribed species. Although the mt16S rRNA gene is commonly used as the standard DNA marker (Vences *et al*., 2005; Sherman & Leaché, 2013), caution is advised against relying solely on a single gene for phylogenetic analysis due to potential inaccuracies (Storfer *et al*., 2009) and lack of signal at varying phylogenetic levels. Lineages or populations are usually recognised as distinct species when the genetic sequence differences exceed the conventional threshold value of 3%, which is considered indicative of species-level divergence in amphibians (Hawlader *et al*., 2016). In fact, recent studies have pointed out inaccuracies in phylogenetic inference and species delimitation caused by the variability in the length of the single 16S gene. As a result, there is growing support for more robust methods based on multiple genes, particularly nuclear

genes (Vences *et al*., 2005). Moreover, combining mtDNA and nuDNA markers enhances the effectiveness of molecular data in various evolutionary analyses (Matsui *et al*., 2014; Zhang & Hewitt, 2003; Dubey *et al*., 2012; Zhang *et al*., 2018; Amanda Estupiñán *et al*., 2023; Yu *et al*., 2009; Lillo *et al*., 2013).

2.5 Bioacoustic communication

Communication strategies are observed across all forms of life, ranging from prokaryotes and plants to fungi and animals (Combarnous & Nguyen, 2020). Organisms engage in communication for various reasons, and the methods they use vary significantly. However, the fundamental components of communication remain consistent across all life forms: the presence of a sender and receiver, along with a signal that is sufficiently detectable and distinct to prevent loss of information or misinterpretation by the receiver (Rendall *et al*., 2009; Lucass *et al*., 2016; Combarnous & Nguyen, 2020; Emmrich *et al*., 2020). Researchers from various disciplines have utilized communication signals to explore behavioural and ecological questions, integrating data from these signals into comprehensive taxonomic approaches across a broad spectrum of animal groups, including anurans. For instance, in Southeast Asia, the analysis of male advertisement calls has revealed hidden diversity among morphologically cryptic species frogs of *Limnonectes* from the Philippines, leading to the discovery of a new species, *Limnonectes beloncioi* (Herr *et al*., 2021). Similarly, the study of advertisement calls in the Asian tree toad species complex *Rentapia hosii* from Peninsular Malaysia resulted in the identification of a new species, *Rentapia flavomaculata* (Chan *et al*., 2020a). Acoustic signals serve as the primary mode of communication for most anuran species, generating diverse calls with specific functions such as reproduction, anti-predator behaviour, mistaken sexual

contact, and territorial defence (Kentwood, 2007; Koehler *et al*., 2017; Lopes *et al*., 2020). Among these calls, advertisement calls are emitted predominantly by males during the breeding season to attract females and defend territories. These calls are vital for reproductive isolation and sexual selection, being species–specific and utilized by females to identify potential mates, assess their sexual receptivity, fitness, and make selections (Batista *et al*., 2015; Toledo *et al*., 2015; Koehler *et al*., 2017; Wei *et al*., 2019). Numerous studies have investigated the advertisement calls of anurans, contributing to call descriptions and the evaluation of species' taxonomic status (e.g., Garg & Biju, 2019; Chen *et al*., 2020; Bang *et al*., 2020; Ong & Shahrudin 2022). Well–documented vocal repertoires offer valuable insights into species delimitation, particularly in cases involving cryptic species (e.g., Herr *et al*., 2021; Seger *et al*., 2021; Al-Razi *et al*., 2020; Phuge *et al*., 2020).

2.6 Family Dicroglossidae of Peninsular Malaysia

The Dicroglossidae (Anderson, 1871) comprises genera and species found in tropical and subtropical regions of Asia and Africa, commonly known as "fork-tongued frogs" (O'Shea *et al*., 2012). With 220 species, the family is divided into two subfamilies: Dicroglossinae (Anderson, 1871), housing 200 species across 11 genera, and Occidozyginae (Fei, Ye and Huang, 1990), hosting 20 species within two genera (Frost, Darrel 2024). Over the years, numerous phylogenetic studies have been conducted on this group, ranging from major family revisions to species–level examinations. Previously considered a subfamily within the Ranidae, dicroglossid's status as a family is now firmly established (Dubois, 1992, 2005; Frost, Darrel 2024). These revisions have revealed the discovery of many new species, highlighting the significant underestimation of the potential for speciation within this family. (Mcleod

et al., 2011; Dinesh *et al*., 2015; McLeod *et al*., 2015; Suwannapoom *et al*, 2016; Herr *et al*., 2021; Yodthong *et al*., 2019, 2021; Pham *et al*., 2022).

In Peninsular Malaysia, two genera from the subfamily Occidozyginae are currently recognized: *Ingerana* (Dubois, 1987) and *Occidozyga* (Kuhl and Van Hasselt, 1822). The genus *Ingerana* consists of one species *I. tenasserimensis* (Sclater, 1892), which inhabits the primary forests in the northern part of Peninsular Malaysia. Zakaria *et al*., (2014) reported a new locality record of this species from Kuala Gandah in the Krau Wildlife Reserve, Pahang. The genus *Occidozyga* comprises three species found in Peninsular Malaysia: *O. lima* (Gravenhorst, 1892), *O. martensii* (Peters, 1867), and *O. sumatrana* (Peters, 1887). All three species are common throughout the region and are listed as Least Concern by the IUCN.

The majority of genera and species fall under the subfamily Dicroglossinae, with three genera currently recognized in Peninsular Malaysia. *Fejervaraya* (Bolkay, 1915), *Hoplobatrachus* (Peters, 1863), and *Limnonectes* (Fitzinger, 1843). Among them, *Hoplobatrachus* stands out as the least diverse genus in the subfamily, consisting only of one species, *H. chinensis* (Osbeck, 1765). Adults of both sexes are large, typically reaching up to 12 centimetres, with females generally larger than males. This frog is often found in wet markets or sold as pets (Hou *et al*., 2006). *Fejervaraya* has three species in Peninsular Malaysia: *F. cancrivora* (Gravenhorst, 1892) and *F. limnocharis* (Gravenhorst, 1829) both of which are common and widespread and listed as least concern by the IUCN. Another species, *F. pulla* (Stoliczka, 1870) has limited information. Although it was described from Penang Hill, Penang Island well over a century ago (Stoliczka, 1870), Chan *et al*., (2010) noted the identification of this species was unclear, and the type series of specimens could not be located, resulting

in its exclusion from the checklist. It is considered a nomen dubium and might even belong to the genus *Hoplobatrachus* instead of *Fejervarya* (Frost, 2024).

The genus *Limnonectes* Fitzinger, 1843, is a diverse clade that contains 79 recognized species, with 13 species reported to occur in Peninsular Malaysia (Frost 2024). The species found in Peninsular Malaysia include: *L. blythii* (Boulenger, 1920), *L. deinodon* (Dehling, 2014), *L. hascheanus* (Stoliczka, 1870), *L. limborgi* (Sclater, 1892), *L. macrognathus* (Boulenger, 1917), *L. malesianus* (Kiew, 1984), *L. nitidus* (Smedley, 1932), *L. paramacrodon* (Inger, 1966), *L. plicatellus* (Stoliczka, 1873), *L. selatan* (Matsui, Belabut, and Ahmad. 2014), *L. tweediee* (Smith, 1935) and *L. utara* (Matsui, Belabut, and Ahmad. 2014). Most of these species exhibit sexual dimorphism, with males characterized by larger body sizes, broader heads, and enlarged odontoid or "fangs" on the lower jaw compared to females. However, there are notable and unusual secondary sexual characteristics in the males of *L. macrognathus*. The caruncles in these males are species-specific in shape, varying from a low, domed structure lacking a free posterior edge (Aowphol *et al*., 2015) and *L. plicatellus* where the males develop a horn-like knob on top of its head (Lambertz *et al*., 2014). Members of these species demonstrate morphological similarity within the group, and some proposed but not yet confirmed species have been discovered in past decade (Chan *et al*., 2014; Matsui *et al*., 2014; Matsui & Nishikawa 2014).

Tadpole development of these species is also remarkably diverse, ranging from exotrophic to endotrophic development in terrestrial nests. While most of the species develop normally such as *L. blythii, L. malesianus, L. nitidus*, *L. plicatellus* and *L. tweediee*, have free-swimming tadpoles relying on external food sources, there are exceptions. The tadpoles of *L. deinodon* are free-swimming but endotrophic, meaning they do not feed but rely on internal stored yolk until metamorphosis into frogs (Ming,

2005). Interestingly, the larvae of *L. hascheanus* and *L. limborgi* were initially believed to undergo direct development. Taylor (1962) and Ohler (1999) reported the larval development of *L. hascheanus*, but unfortunately, detailed descriptions of its ontogenetic transformations are lacking. Boulenger (1920) had proposed that *L. limborgi* exhibited direct development, characterized by enlarged, non-pigmented eggs. However, Rowley & Altig (2012) revealed the nidicolous development (eggs oviposited terrestrially and larvae are free-living but non-feeding) pattern of *L. limborgi*, suggesting that *L. hascheanus* may also have the same larvae development. In addition, it is worth noting that the larval development of some species within the genus remains unknown and further studies will contribute to our understanding of the ecological dynamics of these species as well.

2.7 *Limnonectes hascheanus–limborgi* complex

Limnonectes hascheanus and *Limnonectes limborgi* are two very similar and closely related species, often referred to as the *Limnonectes hascheanus–limborgi* complex. The most recent taxonomy suggests that the number of species in this group might be underestimated within Southeast Asia amphibians (Inger &Stuart 2010; Köhler *et al*., 2021; Huang *et al*., 2022, Garg *et al*., 2022)

Limnonectes hascheanus was originally described by Stoliczka (1870) based on specimens from Penang Island, Malaysia. The author provided some distinctive morphological features such as toes about one-third webbed and large and compressed inner metatarsal tubercle. He observed that this small species (15/16 inch in body size) was commonly found throughout the higher forest (approximately 833m above sea level) on the island. Boulenger (1920) gave additional characteristics of the topotypic specimens collected, noting that males lack vocal sacs and enlarged odontoids, female

specimens with SVL=24mm contained enlarged, non-pigmented ova. *Limnonectes limborgi* was described by Sclater (1892) based on a single specimen from Tenasserim, Myanmar with an SVL=24mm and toes only a third webbed. Boulenger (1893) added more distinct features to the species base on specimens collected from Karen Hills, Myanmar, including one–third to one–half webbed toe, inner metatarsal tubercle large and compressed (2/3 to 3/4 to the first toe), male with internal vocal sac and enlarged odontoids in both males and females. Smith (1929) rejected Boulenger's statement, as he could not find any related characters in the specimens labelled at the museum. Smith (1929) concluded that *L. limborgi* was a junior synonym of *L. hascheanus*. However, a study by Inger and Stuart (2010) argued that none of the previous statements by Boulenger (1893) or Smith (1929) about *L. limborgi* were accurate, confirming that *L. hascheanus* and *L. limborgi* are two distinct species by providing molecular and morphological evidence. An updated distribution range of these two species was also presented. The geographic range of *L. limborgi* extends from southern Myanmar north to northern Thailand and Laos, then curves around central Laos, northeastern Thailand, Cambodia, and southern Vietnam.

Köhler *et al*., (2021) revealed the hidden diversity of *Limnonectes* in Myanmar. *Limnonectes limborgi* once thought to be a single species, was later found to consist of multiple species, among which *L. bagoyoma* was identified as distinct. Molecular studies of mitochondrial DNA sequences confirmed the genetic divergences, while bioacoustic studies further demonstrated a clear separation between the species. Geographic distribution also supports this distinction, with *L. limborgi* found in eastern Myanmar and northwestern Thailand, and *L. bagoyoma* in Lower Myanmar. Additionally, Huang *et al*., (2022) reported *Limnonectes liui* as a junior synonym of *L. limborgi*, by providing genomic and morphological evidence. It is also important to

note that the population from Andaman Island that was previously identified as *L. hascheanus* has also been confirmed to be misidentified *Minervarya andamanensis* (Garg *et al*., 2022). The authors state that the occurrence of *L. hascheanus* in Andaman Island should not only be considered erroneous but should be excluded to avoid further confusion.

In Peninsular Malaysia, the species present were thought to only be *L. hascheanus.* Yet, Inger and Stuart (2010) mentioned a series of specimens of *L. limborgi* from Bukit Lanjan, Peninsular Malaysia, suggesting the possibility of a disjunct distribution of the species. Matsui *et al*., (2014) also reported a specimen of *L. limborgi* from Janda Baik, Peninsular Malaysia. This evidence suggests that reports on the occurrence of *L. hascheanus* in some part of Peninsular Malaysia in the literature might be erroneous identifications of *L. limborgi*. Hence, there is a need to re–examine all available material of the two species in Peninsular Malaysia using integrative methods to correctly identify each species and ascertain the accurate distribution of their populations.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study sites

In this study, voucher material (specimens and tissues) of *Limnonectes hascheanus–limborgi* complex collected from Peninsular Malaysia loaned from La Sierra University Herpetological Collection (LSUHC), USA were examined. To ensure comprehensive coverage of the species distribution, we collected additional samples of topotypic specimens of *L. hascheanus* from Penang Island which is the state in which type locality occurs. Additional samples were obtained from Genting Highlands and Fraser's Hill in Pahang state, where there are doubtful records of *Limnonectes hascheanus* and a lack of voucher material (Figure 3.1 & Appendix B). The voucher specimens were deposited at Universiti Sains Malaysia Herpetological Collection (USMHC). Comparative material was loaned from other institutions, and their abbreviations are as follow: AAH, Amirrudin Ahmad Herpetofauna collection, Universiti Malaysia Terengganu, Terengganu; and ESHQ, Evan S.H. Quah field series. Meanwhile, we also acquired male advertisement calls from all three locations.

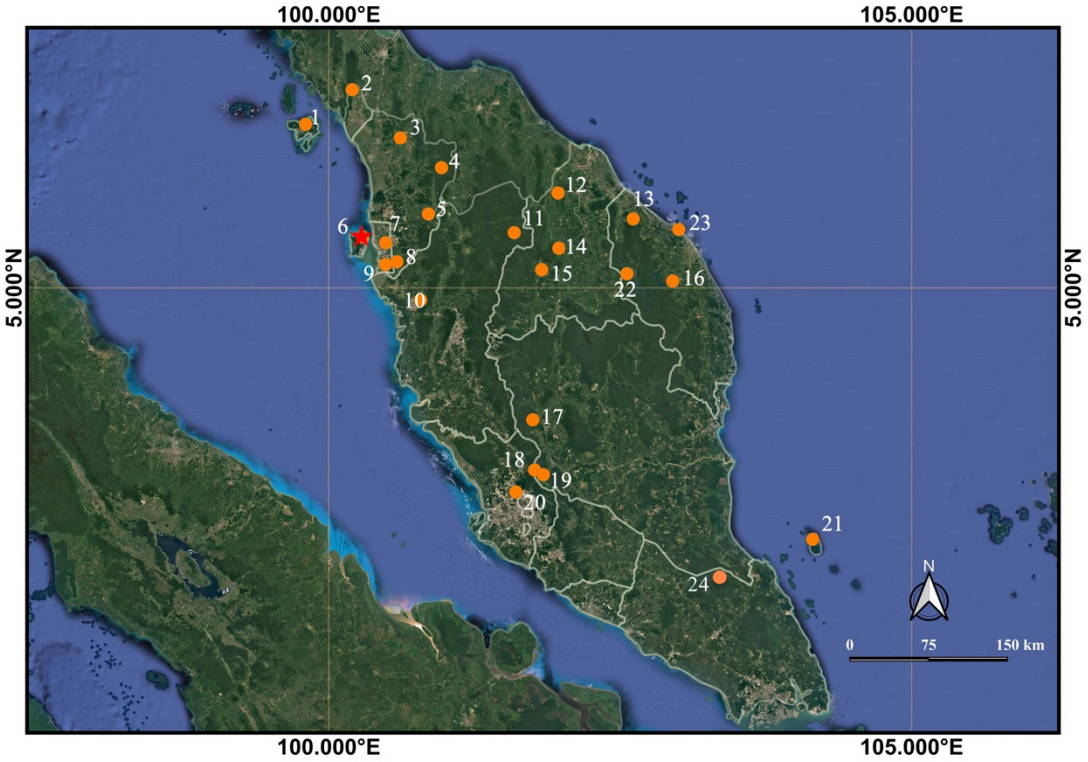

Figure.3.1 Distribution of *Limnonectes hascheanus–limborgi* complex in Peninsular Malaysia. Red star = type locality of *L. hascheanus* at Penang Island, Penang. (1): Langkawi Island, Kedah; (2): Perlis State Park, Perlis; (3): Kuala Nerang, Kedah; (4): Ulu Muda, Kedah; (5) Gunung Inas, Kedah; (6) Penang Island, Penang; (7) Bukit Mertajam, Penang; (8) Batu Hampar, Kedah; (9) Bukit Panchor, Penang; (10) Bukit Larut, Perak; (11) Temengor Forest, Perak; (12) Jedok Forest Reserve, Kelantan; (13) Gunung Tebu, Terengganu; (14) Gunung Stong, Kelantan; (15) Sungai Betis Forest Reserve, Kelantan; (16) Sekayu Lowland Forest, Terengganu; (17) Fraser's Hill, Pahang; (18) Genting Highlands, Pahang; (19) Janda Baik, Pahang; (20) Bukit Lanjan, Selangor; (21) Tioman Island, Pahang; (22) Bukit Maras, Terengganu; (23) Tembat Forest Reserve, Terengganu; (24) Endau Rompin National Park, Johor.

3.1.1 Penang Botanical Garden

The Penang Botanical Garden (Figure 3.2), also known as the Waterfall Gardens, is a public park situated on Jalan Air Terjun on Penang Island. Established in 1884 during the colonial period, it is the oldest garden in Malaysia (Jones 1998; Othman *et al*., 2015). Today, the garden serves not only as a public recreational park but also plays a vital role in conservation and education efforts, both locally and internationally. Its charm is enhanced by lush greenery, plant houses, streams, and a

network of hiking paths, making it a cherished local park and a popular tourist destination (Othman *et al*., 2015).

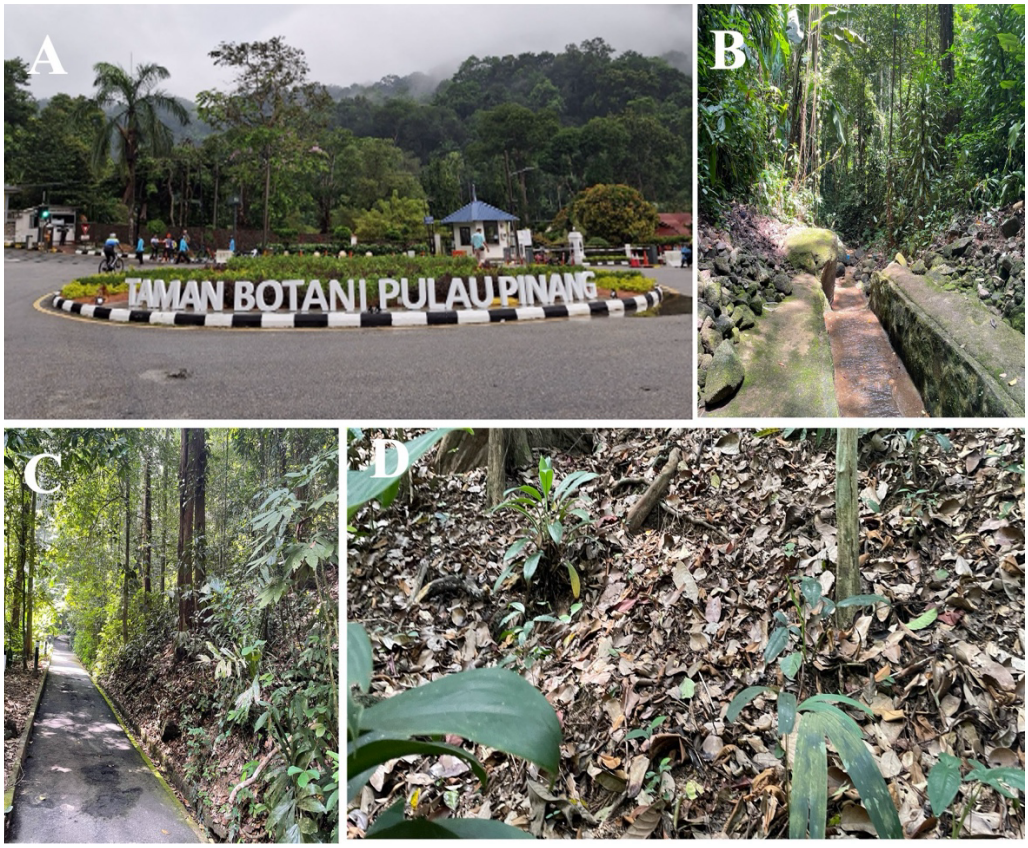

Figure 3.2 Penang Botanic Garden, Penang Island: (A) main entrance of Penang Botanic Garden; (B–C) Lily pond trail: sampling site of the candidate species; (D) natural habitat of the candidate species. Photograph by Zou Bei.

3.1.2 Genting Highlands

Genting highlands (Figure 3.3), situated on the border between Pahang and Selangor within the Titiwangsa range, includes peaks such as Gunung Ulu Kali and Gunung Purun. Once a pristine forest accessible only by jungle trekking, Gunung Purun has become one of Malaysia's most disturbed forests, with much of it now occupied by hotels and an amusement park complex (Ng *et al*., 2012; Peh *et al*., 2011; Musthafa & Abdullah, 2019).

Despite this transformation, Genting Highlands remains ecologically significant, supporting a diverse range of wildlife, including 20% of mammal, 21% of

bird, 8% of reptile, and 21% of amphibian species found in Peninsular Malaysia. It is particularly important for highland species, hosting 51% of highland mammals, 50% of birds, 34.6% of reptiles, and 60% of amphibians in the region (WWF Malaysia, 2002). Endangered species such as the Serow (*Capricornis sumatrensis*) was found here, along with rare species like the Grey Pygmy Fruit Bat (*Aetholops alecto*), Single-finger Larut Skink (*Lygosoma miodactyla*), and Malayan Porcupine (*Hystrix brachyura*) (Musthafa & Abdullah, 2019). Additionally, Genting Highlands is notable for its herpetofaunal diversity, with 32 frog species, 21 lizard species, and four snake species (Chan *et al*., 2019).

Figure 3.3 Genting Highlands, Pahang: (A) overview of Genting Highlands, picture taken from Chin Swee Cave Temple; (B) Jalan Meranti: sampling site of the candidate species; (C) natural habitat of the candidate species. Photograph by Hong Zijia.

3.1.3 Fraser's Hill

Fraser's Hill (Figure 3.4), also known as Bukit Fraser, is a highland area located northeast of Kuala Lumpur, straddling the border between the Pahang and Selangor states in Peninsular Malaysia (Norhayati *et al*., 2011; Er *et al*., 2013). With an altitude ranging from 1000–1525 meters above sea level, it is part of the Titiwangsa Range, which extends north-south through the Malay Peninsula. The area is predominantly covered by tropical lower montane forest, featuring tree species from the *Fagaceae* (oak) and *Lauraceae* (cinnamon) families (Er *et al*., 2013). Fraser's Hill experiences a tropical climate, with mean daily temperatures ranging from 17–25°C and annual rainfall of 3230mm (Norhayati *et al*., 2011).

Notably, the mountainous terrain has gained acclaim for its impressive bird diversity, with records by Strange (2004) documenting an exceptional 247 confirmed bird species and an additional 61 hypothetical species. This avian richness further enhances the allure of Bukit Fraser, making it a sought–after destination for nature enthusiasts. Additionally, the latest herpetofauna record from Fraser's Hill, reported by Chan *et al*., (2019), highlights the area's notable biodiversity documenting 26 species of amphibians from the families Icthyophidae, Bufonidae, Dicroglossidae, Megophryidae, Microhylidae, Ranidae and Rhacophoridae, and 56 species of reptiles from the families Agamidae, Gekkonidae, Scincidae and Colubridae, and others. This thriving population emphasizes the ecological richness and significance of Fraser's Hill as a habitat for diverse wildlife.

Figure 3.4 Fraser's Hill, Pahang: (A) Street view of Jalan Gap–Bukit Fraser's, Fraser's Hill; (B–C) sampling site of the candidate species; (D) natural habitat of the candidate species. Photograph by Hong Zijia.

3.2 Sampling approaches

3.2.1 Field survey and sample collection

Fieldwork was conducted between January 2019 to June 2020 (APPENDIX C). In the field, search efforts were made both day and night. During the daytime, visual encounter surveys were carried out along the forest trail and surrounding sampling sites. Also, the calling males were targeted to pinpoint the possible locations, and those places were revisited at night. Collections were mainly done at night by locating the calling males, but active searches were also done opportunistically during the day. Specimens were caught by hand and kept in plastic Ziploc bags with some

leaf litter. Notes about the microhabitat and observations of other calling males heard nearby were also recorded. Photographs of the live specimens were taken for morphology reference.

3.2.2 Specimen preservation and storage

Collected individuals were preserved following the protocols following Hong *et al*., (2021) and Quah *et al*., (2021): the specimens underwent euthanasia using Tricaine (Ethyl 3–aminobenzoate methane sulfonate) followed by fixation in 10% formalin (Simmons 2015). They were then transferred to a 70% ethanol solution for storage, while liver tissue samples were preserved in 100% undenatured ethanol. The preserved specimens have been deposited at the Universiti Sains Malaysia Herpetological Collection (USMHC) for future reference.

3.2.3 DNA extraction and PCR amplification

3.2.3(a) DNA extraction

In this study, a total of forty–eight liver tissue samples were used for genomic DNA extraction. Forty–three of these tissue samples were loaned from La Sierra University (LSUHC), with the additional samples collected during fieldwork at Fraser's Hill and Genting Highlands (USMHC). The protocol for DNA extraction mainly followed Yari *et al*., (2017), with slight adjustments made due to different voucher material and laboratory conditions. The protocols were as follows:

1) A tiny fraction (about a sesame seed-sized amount was sufficient) of liver tissue was cut from the preserved tissue sample and transferred into a 2ml Eppendorf tube labelled with an identification number.

2) 1ml tissue digestion buffer (10mM Na_2EDTA, 20mM Tris–HCL and 100mM NaCl (pH 7.4-8.0) was added and mixed with a vortex for breakup and rinsing.

3) 100µl of 10% SDS and 20µl of proteinase K were added.

4) Microtube was incubated at 55°C for 4–8 hours until tissue was fully dissolved.

5) It was centrifuged in 5000rpm for 10min and then the supernatant was removed.

6) 350µl of chloroform and 350µl of saturated NaCl (5M) were added and then the microtube was put into ice tray and placed in a -20°C freezer for 5min.

7) Microtube was shaken for 30sec manually and centrifuged at 5000rpm for 10min at 4°C.

8) The upper phase (about 1ml or less) was transferred into a new 2ml microtube and 1ml of cold absolute ethanol and 150ul of 5M ammonium acetate were added.

9) The tube was gently shaken; in successfully extractions, the DNA appeared as a white or semi–translucent skein in this step.

10) Microtube was centrifuged for 10min at 13000rpm at 4°C.

11) Supernatant was removed, and the pellet was washed with 1ml of chilled 70% ethanol and centrifuged at 10000rmp for 10min.

12) Supernatant was removed, and the pellet was left at room temperature to be dried.

13) About 50–100µl of ddH_2O was added to dissolve the DNA pellet and the final DNA solution was stored at -20°C for further use.

3.2.3(b) Agarose gel electrophoresis

The agarose gel (1%) electrophoresis was used to estimate and confirm the final product of extracted DNA.