

**MOLECULAR PHYLOGENY AND GENETIC  
DIVERSITY OF ROUNDLEAF BATS  
(HIPPOSIDERIDAE: *HIPPOSIDEROS*)  
IN SABAH, MALAYSIA.**



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UNIVERSITI MALAYSIA SABAH

**INSTITUTE FOR TROPICAL BIOLOGY AND  
CONSERVATION**

**UNIVERSITI MALAYSIA SABAH**

**2022**

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CONSERVATION  
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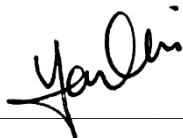
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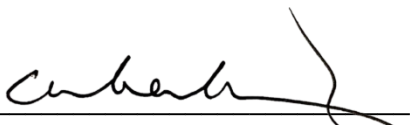


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DEGREE : **MASTER OF SCIENCE**  
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A handwritten signature in black ink, appearing to read 'C. H. Hasan', is written over a horizontal line.

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Lok Yen Chi  
17 May 2022

## ABSTRACT

*Hipposideros* spp. or roundleaf bats is one of the most common taxonomic unit found in the understorey layer of Bornean lowland forests. Being a group of insectivorous bats, *Hipposideros* spp. provides essential service as an ecological indicator. In spite of the valuable services they provide to the natural ecosystem, the phylogenetic status of this genus is still eluded with uncertainties, which may hinder effective management of this genus. A good resolution of phylogeny is needed for taxonomic clarification, conservation management and zoonotic outbreak predictions. Besides that, the availability of genetic diversity assessment of *Hipposideros* spp. is still limited. Considering the importance of genetic diversity on a species' survivability during unprecedented events, on top of the cruciality of phylogeny, this study aims to achieve two objectives, which are to (1) To determine the phylogenetic relationships between five species of roundleaf bats (*Hipposideros* spp.) in Sabah using two mitochondrial and one nuclear markers and (2) assess the genetic diversity of the four populations of *Hipposideros cervinus* in Sabah using two mitochondrial gene regions. Field samplings were conducted across several protected areas in Sabah – Crocker Range Park, Tawau Hills Park, Gomantong Forest Reserve, Maliau Basin Conservation Area and Danum Valley Conservation Area. Three genes; Cytochrome b (cyt b) (1140 bp), Recombination Activating Gene (RAG 1) (1086 bp) and Displacement loop (D-loop) (1600 bp) were concatenated for phylogenetic analyses, whereas for *H. cervinus* genetic diversity assessment, only the concatenated matrix of the two mitochondrial genes were utilised. Cyt b gene tree presented three main lineages of *Hipposideros* species found distributed in Sabah, and reaffirm the polyphyletic placement of several *Hipposideros* spp. Despite so, Cyt b gene tree itself did not receive high degree of nodal support. On the contrary, the phylogenetic tree resulted from the concatenation of Cyt b, D-loop and RAG 1 provided much better resolution, with most of the nodes getting full support. It is revealed that the specimens analysed belongs to two species groups – the large bodied diadema species group and the smaller bodied, more speciose, bicolor species group. Supplementary genetic divergence analysis provide evidence to corroborate the presence of species complex within the currently recognised *H. ater*. Notable intraspecific structuring observed within the *H. cervinus* of Malaysian Borneo characterised with high genetic diversity and positive gene flow between the different populations. This unconventional pattern of high genetic variability with significant degree of gene interchange can be explained by the slow reproductive rate and long generation time of Chiroptera. Ultimately, this study has managed to provide information on the phylogenetic relationships within the genus *Hipposideros* in Sabah, as well as on the status of genetic diversity status of *Hipposideros cervinus*. From a conservation view-point, these are useful in aiding conservation planning and management of the leaf-nosed bats of Sabah, which can incorporate molecular data rather than only relying on the conventional data.

## **ABSTRAK**

### **FILOGENI MOLEKULAR DAN KEPELBAGAIAN GENETIK KELAWAR LADAM BULAT (HIPPOSIDERIDAE: HIPPOSIDEROS) DI SABAH, MALAYSIA**

*Hipposideros* spp. atau kelawar ladam bulat merupakan satu unit taksonomi yang boleh dijumpai dalam lapisan bawah hutan tropika Borneo. *Hipposideros* spp. merupakan kumpulan kelawar pemangsa serangga yang memberikan khidmat penting sebagai indikator ekologi. Walaupun demikian, status filogeni genus ini masih belum dapat dipastikan. Hal ini berkemungkinan akan menghalang pengurusan yang efektif bagi genus ini. Resolusi filogeni yang baik diperlukan untuk pengesahan taksonomi, pengurusan pemuliharaan dan ramalan penularan zoonotik. Selain itu, penilaian kepelbagaian genetik bagi *Hipposideros* spp. adalah terhad. Mengambil kira kepentingan kepelbagaian genetik terhadap kebolehamandirian sesuatu spesis, kajian ini bertujuan untuk mencapai dua objektif, iaitu (1) untuk mengenalpasti hubungan filogenetik di antara lima spesis kelawar ladam bulat (*Hipposideros* spp.) di Sabah menggunakan dua gen mitokondria dan satu gen nuclear, dan (2) menilai kepelbagaian genetik untuk empat populasi *Hipposideros cervinus* di Sabah menggunakan dua gen mitokondria. Persampelan di lapangan telah dilakukan di beberapa kawasan terlindung Sabah – Banjaran Crocker, Taman Bukit Tawau, Hutan Simpan Gomantong, Kawasan Konservasi Maliau Basin dan Lembah Danum. Tiga gen; Sitokrom b (cyt b), *Recombination Activating Gene* (RAG 1) dan *Displacement loop* (D-loop) telah digabungkan untuk analisis filogeni, manakala untuk penilaian kepelbagaian genetik *H. cervinus*, hanya dua gen mitokondria yang digabungkan. Filogeni untuk gen sitokrom b mendedahkan tiga kumpulan *Hipposideros* yang terdapat di Sabah, dan mengesahkan semula status polifiletik untuk beberapa spesis *Hipposideros*. Walaubagaimanapun, pokok filogeni untuk gen sitokrom b tidak menerima nilai sokongan yang tinggi. Sebaliknya, pokok filogeni yang terhasil daripada penggabungan gen sitokrom b, D-loop dan RAG1 memberikan resolusi yang lebih baik, dengan kebanyakan nod menerima kebarangkalian posterior yang penuh. Hal ini juga mendedahkan bahawa spesis yang diperangkap untuk kajian ini adalah terdiri daripada dua kumpulan spesis – kumpulan berbadan besar, diadema, dan kumpulan berbadan kecil iaitu kumpulan bicolor. Analisis tambahan bagi jarak genetik memberikan bukti yang menyokong kewujudan spesis kompleks dalam spesis *H. ater*. Terdapat juga struktur genetik yang ketara dalam kalangan populasi *H. cervinus* di Malaysia Borneo, di mana kepelbagaian genetik adalah tinggi dan disertai dengan aliran gen yang ketara. Keadaan ini dapat dijelaskan dengan kadar pembiakan kelawar yang perlahan serta masa generasi yang panjang. Secara ringkasnya, kajian ini telah dapat memberikan maklumat filogeni bagi genus *Hipposideros* di Sabah, di samping memberi gambaran tentang status kepelbagaian genetik untuk *Hipposideros cervinus*. Daripada segi pemuliharaan, maklumat-maklumat ini adalah berguna untuk membantu dalam perancangan konservasi dan pengurusan kelawar ladam bulat di Sabah.



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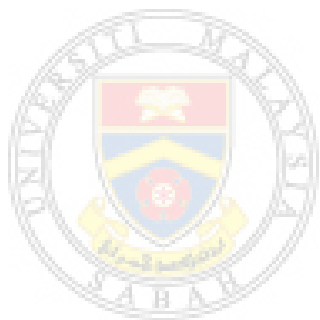
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## LIST OF ABBREVIATIONS

<b>IUCN</b>	-	International Union for Conservation of Nature
<b>cyt b</b>	-	Cytochrome b
<b>RAG1</b>	-	Recombinant Activating Gene 1
<b>D-loop</b>	-	Displacement loop
<b>DNA</b>	-	Deoxyribonucleic Acid
<b>PCR</b>	-	Polymerase Chain Reaction
<b>m a.s.l.</b>	-	metre above sea level
<b>lbs.</b>	-	pound
<b>g</b>	-	gram
<b>ddH<sub>2</sub>O</b>	-	Double distilled water
<b>U</b>	-	Unit
<b>sp.</b>	-	species (singular)
<b>spp.</b>	-	species (plural)
<b>Hd</b>	-	Haplotype diversity
<b><math>\pi</math></b>	-	Nucleotide diversity
<b>h</b>	-	Number of haplotypes
<b>ML</b>	-	Maximum Likelihood
<b>BI</b>	-	Bayesian Inference
<b>NJ</b>	-	Neighbour Joining
<b>MP</b>	-	Maximum Parsimony
<b><math>\mu</math>L</b>	-	Microlitre
<b>°C</b>	-	Degree Celsius
<b>mM</b>	-	Millimolar
<b><math>\mu</math>M</b>	-	Micromolar
<b>MgSO<sub>4</sub></b>	-	Magnesium Sulphate
<b>dNTP</b>	-	Deoxynucleoside triphosphate
<b>FA</b>	-	Forearm

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# CHAPTER 1

## INTRODUCTION

### 1.1 General Introduction

Bats, or taxonomically known as Chiroptera, is the second largest mammalian order in the world. Chiroptera can be found thriving in almost every type of ecosystems, with up to 1 500 species worldwide and increasing (Wilson, 2020; Scherrer *et al.*, 2019). The tropical region is where bats found to be most abundant due to its stable and warm climate (Furey & Racey, 2016; McKay, 2006). To be specific, in Southeast Asia alone, 330 bats species are recorded, accounting for up to one third the region's overall mammalian composition.

This speciose group of volant mammals can be broadly segregated into Microchiroptera (insectivorous bats) and Megachiroptera (frugivorous and nectarivorous bats), in which it is estimated that two third of the entirety of bat species of the World belongs to the former (Kunz *et al.*, 2011). Microchiropterans specifically has been known to contribute to arthropod suppression, including crop pests as a result of their dietary consumptions. They have been documented to prey on insects like Lepidoptera, Diptera and Coleoptera (Kunz *et al.*, 2011). In addition to that, another vital ecosystem service provided by Microchiroptera is that they aid in distributing nutrients to areas that are deprived of them. This is done through nitrogen and phosphorus rich guano excretion mid-flight (Kunz *et al.*, 2011).

Moreover, insectivorous bats are sensitive to changes, be it in the form of temperature changes or landscape alterations. To elaborate, the changes in their abundance and mortality rate can reflect abnormalities in their habitat, such as unhealthy level of heavy metal pollutants (Kasso & Balakrishnan, 2013), possible

emergence of zoonoses (Jones *et al.*, 2009) and the decline of insect populations. In addition, it is also relatively easy to monitor their populations in comparison to other mammals and being widely distributed (Jones *et al.*, 2009) makes them effective bioindicators. In a sense, the measurable parameters of a bat populations can be extrapolated to estimate the effect of such changes to a larger scale or other biotas.

In spite of the essential ecological roles that these microchiropterans has to the well-being of the ecosystem, their evolutionary histories, particularly Hipposideridae, one of the microchiropteran families in Southeast Asia with the highest uncertainty (Kingson, 2010). The knowledge on the phylogeny of a species is particularly useful for conservation and management of a taxa and the ecosystem as a whole. By investigating into such topics, vague taxonomic status of a species can be clarified (Byrne, 2003). Plus, information from phylogenies can be extracted to identify rare and widespread species, sorting out conservation priorities (Byrne, 2003). Furthermore, the size of past populations (Page & Holmes, 2008) and the historical biogeography distribution of a species (Swenson, 2019) can be inferred from a well-studied phylogeny of a species. Most importantly, phylogenetic studies give scientist the data to decide on the direction on long term conservation, management, and monitoring planning. Phylogeny is an essential tool to predict the possible response of a species towards environmental dynamics (Lean & Maclaurin, 2016; Cavender-Bares *et al.*, 2009) by aligning historical events with the molecular clock of the taxa. Understanding and preserving phylogenetic information also safeguard features and services that might be useful for future events (Yessoufou & Davies, 2016), for example, preventing the loss of rare taxa that has specific ecological roles.

To elaborate further, multiple studies have attempted to resolve the intrageneric relationship of *Hipposideros* spp., one of the main genus of Hipposideridae. Unfortunately, no unanimous resolutions have been reached and the grouping within this genus was still being reshuffled recently (Patterson *et al.*, 2020; Monadjem, 2019). In fact, phylogenies resulted from the usage of morphometrics (Bogdanowicz & Owen, 1998; Hill, 1963), do not agree with those produced using molecular markers (Patterson *et al.*, 2020, Murray *et al.*, 2012).

Equivalently, it is also important to look into the genetic diversity of *Hipposideros cervinus*, a species of Hipposideridae. This is due to as of date, not much is known about the genetic diversity status of this species, despite it being one of the most common Hipposiderid found in the forest underlayer of tropical lowland forests (Phillipps & Phillipps, 2016). The understanding the magnitude of genetic diversity a specific species has is also of the same importance as the knowledge on the evolutionary relationships between species. For a species to survive a treacherous environmental threats like disease outbreak or extreme climate, genetic variation allows adaptive differentiation to happen (Booy *et al.*, 2000), hence preventing extinction.

## 1.2 Justification of Study

Several studies have attempted to resolve the phylogenetic relationships among the members of this genus produced statistically weak resolutions and, in some cases, were plague with polytomies (Foley *et al.*, 2014; Murray *et al.*, 2012; Sazali *et al.*, 2011). This is further supported by the statement made by van den Bussche & Lack (2013), who pointed that the phylogeny researches below the familial taxonomic classification in regards to Chiroptera is still very much lacking. On top of the unresolved phylogeny, multiple studies also discovered evidence pointing to the existence of species complex within the genus *Hipposideros* (Foley *et al.*, 2017; Murray *et al.*, 2012; Shepherd & Shepherd, 2012; Francis *et al.*, 2010).

Albeit there are several studies (Murray *et al.*, 2012; Sazali *et al.*, 2011) that have explored into the phylogeny of *Hipposideros* of Malaysia, the involvement of specimens collected from Sabah in these studies are relatively few in comparison with samples from other parts of the country due to the limited availability of genetic sequences from this specific locality (Sabah) in Genbank, and the limited resources that restricts extensive field samplings. With respect to how Borneo is one of the major evolutionary hotspot (de Bruyn *et al.*, 2014), this may underestimate or overlook the unique evolutionary lineages that could present in Sabah. This also highlights how much the study of evolutionary history of *Hipposideros* is lagging

behind, particularly in Sabah, in spite of genetics being one of the resources protected and managed in the Sabah Biodiversity Strategy 2012-2022 (Sabah Biodiversity Strategy, 2012). Likewise, the International Union for Conservation of Nature (IUCN) also recognises genetic resources as one of the three key items for biological diversity conservation (Fernández-Gracia, 2017). Hence, this study is attempting to fill this gap by focusing on collecting specimens of *Hipposideros* in Sabah.

From zoonotic point of view, a well-established phylogeny can potentially forecast an outbreak before it happens by providing insights to the persistency and reproductive ability of zoonoses (Foley *et al.*, 2014). A meta-study by Guy *et al.* (2019) found that for bats, phylogeny does influence the amount of viral diversity hosted by these zoonotic reservoirs. To further emphasize the importance of this study is that a preliminary study (Ar Gouilh *et al.*, 2011) has detected the presence of betacoronaviruses-b in *H. larvatus*, a species that is distributed in Borneo and has found preliminary evidence that betacoronaviruses in Rhinolophidae is a spillover from Hipposideridae. Since the virulent materials hosted by Hipposideridae is still relatively unknown (Foley *et al.*, 2014), establishing a good phylogeny will be a decent initiation point, as it can serve as the basis for virus diversity estimation.

On another front, it is essential for a species to have a diverse gene pool to increase the species' odds at surviving threats of various kind (Booy *et al.*, 2000). Plus, considering how allelic loss has also been recorded in a species of insectivorous bats (*Kerivoula papillosa*) in Peninsula Malaysia due to habitat fragmentation (Struebig *et al.*, 2011), it is rational to at least conduct a preliminary assessment on the genetic variability of a *Hipposideros* sp., as such information is yet to be available at the moment.

As per the above justifications, this study aims to resolve the phylogenetic relationship within *Hipposideros*, as well as to obtain preliminary information on the genetic diversity status of *Hipposideros cervinus*. The sampling scope of this study is the state of Sabah, Malaysian Borneo. Overall, these are in accordance to the suggestions by Mohd-Ridwan *et al.* (2018) and Francis *et al.* (2010) who accentuates

the significance of genetic data inclusion in wildlife conservation planning, execution, and monitoring.

### **1.3 Objectives**

- i. To determine the phylogenetic relationships between five species of roundleaf bats (*Hipposideros* spp.) in Sabah using two mitochondrial and one nuclear markers.
- ii. To assess the genetic diversity of the four populations of *Hipposideros cervinus* in Sabah using two mitochondrial gene regions.



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## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 *Hipposideros* spp. of Borneo.

The genus *Hipposideros* belongs to the family Hipposideridae. It has been suggested that this family first appeared during Eocene, with the oldest fossil discovered in Europe, pointing to a mid-Eocene origin (Vaughan *et al.*, 2011). What sets them apart from the other families, particularly Rhinolophidae are their unique noseleaf, as well as the features of the fingers attached to their wings. Unlike the other families, bats from this family possess only two phalanges in their fingers, rather than three, and for the second finger, only metacarpal without any phalanges can be observed. Their ears are also medium in size, where tragus is absent (Wilson, 2020; Phillipps & Phillipps; 2016). Hipposideridae is found to be the most widespread and flourished species in a wide range of ecological conditions (Murray *et al.*, 2012). In the tropics, similar to Rhinolophidae and Vespertilionid, hipposiderids dwell in the interior part of the forest, where they usually forage and hunt close to the ground. In terms of acoustics, they are rather well developed, where its echolocation consists of constant frequency and ends with a short duration of downsweep (Hall, 1989).

Within Hipposideridae, there are nine recognised genera, including *Hipposideros* itself (Vaughan *et al.*, 2011; Nowak & Walker, 1994). Preceding to the nomenclature review made by John Gray in 1831, this genus was initially known as *Rhinolophus*, albeit there have been proposals to rename this genus as *Phyllorrhina*. Gray was the first to acknowledge the noseleaf features of this genus, hence, proposed the generic name, *Hipposideros* (Hill, 1963). Within *Hipposideros*, 9 species groups have been established based on morphological similarities, where 4 of them

are distributed on the island of Borneo, particularly *bicolor*, *diadema*, *galeritus* and *larvatus* species group, with *bicolor* being the most speciose group (Simmons, 2005).

It should be noted that according to Simmons (2005), Hipposideridae does not have any subfamily. In terms of facial morphology, Hipposiderids have intricate skin outgrowth on their muzzle, which resembles a leaf (Figure 2.1). The anterior of their noseleaves are horseshoe-like, in which tiny, additional leaflets may present, depending on the species (Nowak & Walker, 1994; Hill, 1963). Besides, this ecologically diverse family of insectivorous bats have some other prominent features, such as having only 2 toe bones, rather than 3, the lack of tragus and sella, as well as small eyes but large ears (Vaughan *et al.*, 2011; Nowak & Walker, 1994; Hill, 1963). Hipposiderids are highly adapted to foraging in cluttered surroundings, as such low aspect ratio of the wing (Lee *et al.*, 2012; Struebig *et al.*, 2012; Hall, 1989) that supports navigation in forest interiors. Furthermore, to support the incessant take-off and landing during foraging, the pelvic of hipposiderids is enlarged at the anterior parts (Vaughan *et al.*, 2011).

In Borneo, a total of 11 *Hipposideros* spp. are present (Phillipps & Phillipps, 2016). *Hipposideros* is distinguished from the other genera of Hipposideridae as the species of this genus have controllable, wax-like substance secreting sac at the back of its noseleaf (Nowak & Walker, 1994). It is also characterised by the vertical septa on its posterior noseleaf (Figure 2.1) (Phillipps & Phillipps, 2016). The dentistry of *Hipposideros* spp. is also unique. The premolars at the upper front are inconsistently reduced and the upper molars are heavy but have simple structure (Francis, 2019). In Borneo, it is the most commonly found genus of bats in the forest understorey, sharing the same foraging guild as *Kerivoula* and *Rhinolophus* (Phillipps & Phillipps, 2016). To catch its prey, *Hipposideros* fly lower than most bats, approximately 1-2 meters above the forest floor (Hall, 1989) and gets back to its roost before ingesting its prey (Nowak & Walker, 1994). For Bornean *Hipposideros*, roosting is generally grouped into two types, either in large colonies in cave systems, or solitary roosting in places like tree hollows (Phillipps & Phillipps, 2016).

Generally, some of the *Hipposideros* spp. distributed in Borneo have consistently been placed in the same species grouping irrespective of the revisions done over time. This include *H. diadema* and *H. doriae* (Table 2.1), who remained in the diadema and bicolor group respectively, even though the diagnostic tool have switched from morphology as in the cases of Tate (1914) and Hill (1963) to molecular in Murray *et al.* (2012), Monadjem (2019) and Patterson *et al.* (2020).

The bicolor species groupings presented by Simmons (2005) are similar to those determined using molecular data, as in Patterson *et al.* (2020) and Murray *et al.* (2012), at least for the species focused in this study. With the inclusion of nuclear introns in their study, the species group findings in Patterson *et al.* (2020) is consistent to Murray *et al.* (2012), whom only employed data from one mitochondrial and one nuclear genes. On the contrary, Monadjem (2019) segregated some of the species belonging in the bicolor group into other species groups, as such, *H. ater*, *H. cineraceus*, *H. ridleyi*, *H. dyacorum* and *H. bicolor* into the ater subgroup and placed *H. cervinus*, *H. coxi* and *H. galeritus* into the calcaratus species group, which was later demoted into their original group (Bicolor) by Patterson *et al.* (2020).

Furthermore, the notion of species complexes also resonates in not one but five of the *Hipposideros* spp. found on Borneo. Csorba *et al.* (2008) stated that the widely distributed *H. diadema* might be comprised of several distinct species, but they did not present the data used to make such claim. Meanwhile, Francis (2019) and Son *et al.* (2019) both agree that *H. larvatus* is comprised of a species complex. Son *et al.* (2019) backed their statement using the basis of echolocation range, and this then was further strengthened by Francis (2019), who used body size to supplement acoustic data. Likewise, *H. ater* is potentially another species complex, as presented by Douangboupha *et al.* (2011) and Douangboupha *et al.* (2010b). Both studies came to such conclusion with the aid of morphometric and echolocation data.