



Faculty of Medicine and Health Sciences

**Investigating the Phylogenetic Diversity of SARS-CoV-Like Viruses in
Various Species of Bats in Sarawak, Malaysia**

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Investigating the Phylogenetic Diversity of SARS-CoV-Like Viruses in Various Species of Bats in Sarawak, Malaysia

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Bats are natural reservoirs for a diverse range of coronaviruses (CoVs), including those closely related to SARS-CoV and SARS-CoV-2. Their unique ecological traits, including high mobility, diverse dietary habits, and social roosting behavior, facilitate viral maintenance and transmission, making them critical subjects for understanding zoonotic spillover risks. Despite Malaysia's rich bat diversity, particularly in Sarawak, studies on bat CoVs (BtCoVs) remain limited. This study aims to fill this gap by investigating the prevalence, diversity, and phylogenetic relationships of bat CoVs in western and northern Sarawak, Malaysian Borneo. A total of 346 fecal samples were collected from 29 bat species spanning six families, including Pteropodidae, Hipposideridae, Vespertilionidae, Miniopteridae, Nycteridae, and Rhinolophidae. To ensure comprehensive detection, two widely used molecular assays, the Quan (Q-assay) and Watanabe (W-assay) pan-CoV PCR protocols, were employed. The selection of these assays was based on their established sensitivity in detecting both known and potentially novel CoV lineages by targeting conserved regions of the RNA-dependent RNA polymerase (RdRp) gene. The Q-assay has been widely utilized for detecting a broad range of CoVs across various host species, while the W-assay is optimized for bat-associated CoVs, making their combined application suitable for evaluating detection efficacy. The performance of both assays was statistically compared using the Kappa statistic and McNemar's test to assess agreement and detection differences. The results revealed a CoV prevalence of 14.45% with the Q-assay and 12.72% with the W-assay, with a combined prevalence of 22.83%. The two assays demonstrated fair agreement ($\kappa=0.286$, $p<0.001$) and no statistically significant difference in detection capability (McNemar's $p>0.05$), indicating comparable performance in identifying bat CoVs. Phylogenetic analysis of RdRp sequences identified six distinct clades within

alphacoronaviruses (α -CoVs) and *betacoronaviruses* (β -CoVs), including two unclassified *Borneo- α -CoVs* and four classified CoVs within the subgenera *Minunacovirus*, *Rhinacovirus*, *Nobecovirus*, and *Sarbecovirus*. Notably, *Sarbecovirus*-related sequences were detected in Rhinolophidae bats, further supporting their role as key reservoirs of viruses related to SARS-CoV and SARS-CoV-2. The use of two molecular assays provided a more comprehensive picture of CoV diversity and demonstrated the importance of using complementary detection methods. While both assays showed similar detection capabilities, their combined use allowed for the identification of a broader range of CoVs, including potentially novel strains. This study represents the first detailed phylogenetic analysis of bat CoVs in Sarawak derived from fecal samples, addressing a significant research gap. The findings underscore the importance of continued surveillance efforts in high-biodiversity regions to improve understanding of CoV ecology and mitigate zoonotic risks. Future research should explore whole-genome sequencing approaches to further characterize unclassified CoV clades and refine molecular assays for enhanced detection.

Keywords: Bat coronaviruses, Q-assay, W-assay, *Sarbecoviruses*, zoonotic

Menyelidik Kepelbagaian Filogenetik Virus Seakan Sars-CoV dalam Pelbagai Spesies Kelawar di Sarawak, Malaysia

ABSTRAK

Kelawar merupakan perumah semula jadi bagi pelbagai jenis koronavirus (CoV), termasuk yang berkait rapat dengan SARS-CoV dan SARS-CoV-2. Ciri ekologi unik mereka, seperti mobiliti tinggi, tabiat pemakanan yang pelbagai, dan tingkah laku berkoloni, memudahkan pemeliharaan dan penularan virus, menjadikan mereka subjek penting dalam memahami risiko limpahan zoonotik. Walaupun Malaysia, khususnya Sarawak, memiliki kepelbagaian kelawar yang tinggi, kajian mengenai koronavirus kelawar (BtCoV) masih terhad. Kajian ini bertujuan untuk mengisi jurang tersebut dengan menyiasat kelaziman, kepelbagaian, dan hubungan filogenetik BtCoV di bahagian utara dan barat Sarawak, Borneo Malaysia. Sebanyak 346 sampel najis dikumpulkan daripada 29 spesies kelawar yang merangkumi enam famili, termasuk Pteropodidae, Hipposideridae, Vespertilionidae, Miniopteridae, Nycteridae, dan Rhinolophidae. Untuk memastikan pengesanan yang menyeluruh, dua ujian molekul pan-CoV yang banyak digunakan, iaitu protokol PCR Quan (Q-assay) dan Watanabe (W-assay), telah digunakan. Pemilihan ujian ini berdasarkan kepekaan yang terbukti dalam mengesan kedua-dua garis keturunan CoV yang diketahui dan yang berpotensi baharu dengan menyasarkan kawasan terpelihara dalam gen RNA-dependent RNA polymerase (RdRp). Q-assay telah digunakan secara meluas untuk mengesan pelbagai jenis CoV dalam pelbagai spesies perumah, manakala W-assay dioptimumkan untuk CoV yang berkaitan dengan kelawar, menjadikan gabungan kedua-duanya sesuai untuk menilai keberkesanan pengesanan. Prestasi kedua-dua ujian dibandingkan secara statistik menggunakan statistik Kappa dan ujian McNemar untuk menilai tahap kesepakatan dan perbezaan pengesanan. Keputusan menunjukkan kelaziman CoV sebanyak 14.45%

menggunakan *Q*-assay dan 12.72% menggunakan *W*-assay, dengan kelaziman bersama keseluruhan sebanyak 22.83%. Kedua-dua ujian menunjukkan kesepakatan yang sederhana ($\kappa=0.286$, $p<0.001$) dan tiada perbezaan ketara dalam keupayaan pengesanan (ujian McNemar, $p>0.05$), menunjukkan prestasi yang setanding dalam mengenal pasti BtCoV. Analisis filogenetik bagi urutan RdRp mengenal pasti enam klad berbeza dalam alphacoronavirus (α -CoV) dan betacoronavirus (β -CoV), termasuk dua CoV Borneo- α yang tidak diklasifikasikan serta empat CoV dalam subgenera Minunacovirus, Rhinacovirus, Nobecovirus, dan Sarbecovirus. Penemuan jujukan yang berkaitan dengan Sarbecovirus dalam kelawar Rhinolophidae mengukuhkan lagi peranan mereka sebagai perumah utama virus yang berkaitan dengan SARS-CoV dan SARS-CoV-2. Penggunaan dua ujian molekul ini memberikan gambaran yang lebih menyeluruh mengenai kepelbagaian CoV dan menunjukkan kepentingan penggunaan kaedah pengesanan pelengkap. Walaupun kedua-dua ujian menunjukkan keupayaan pengesanan yang serupa, gabungan penggunaannya membolehkan pengenalanpastian spektrum CoV yang lebih luas, termasuk garis keturunan baharu yang berpotensi. Kajian ini merupakan analisis filogenetik terperinci pertama terhadap BtCoV di Sarawak yang diperoleh daripada sampel najis, sekali gus menangani jurang penyelidikan yang ketara. Penemuan ini menekankan kepentingan usaha pemantauan yang berterusan di kawasan berkepelbagaian tinggi untuk meningkatkan pemahaman tentang ekologi CoV dan mengurangkan risiko zoonotik. Kajian masa depan perlu meneroka pendekatan penjujukan genom penuh untuk mencirikan klad CoV yang tidak diklasifikasikan dan memperbaiki ujian molekul bagi meningkatkan kepekaan pengesanan.

Kata kunci: Koronavirus kelawar, *Q*-assay, *W*-assay, Sarbecovirus, zoonotik

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LIST OF ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
AIC	Akaike Information Criterion
ARS	Acute respiratory syndrome
BLASTn	Basic Local Alignment Search Tool nucleotide
bp	Base pairs
BtCoVs	Bat Coronaviruses
cDNA	Complementary DNA
CoVs	Coronaviruses
COVID-2019	Coronavirus Disease of 2019
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPP4	Dipeptidylpeptidase 4
E	Envelope protein
EID	Emerging Infectious Diseases
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide
FASTA	Fast alignment
GTR	General time reversible
HCoVs	Human Coronaviruses
HCoV-229E	Human Coronavirus-229E
HCoV-HKU1	Human Coronavirus-HKU1
HCoV-NL63	Human Coronavirus-NL63
HCoV-OC43	Human Coronavirus-OC43

HE	Hemagglutinin-esterase
ICTV	International Committee on Taxonomy of Viruses
IUCN	International Union for Conservation of Nature
kb	Kilobases
LMICs	Low- and middle-income countries
M	Membrane glycoprotein
MEGA	Molecular Evolutionary Genetics Analysis
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
min	Minutes
ML	Maximum Likelihood
mRNA	Messenger RNA
MUSCLE	Multiple Sequence Comparison by Log-Expectation
N	Nucleocapsid protein
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
nsp	Non-structural protein
NP	National Park
ORF	Open reading frame
PAA	Three-amino-acid
PCR	Polymerase Chain Reaction
PEDV	Porcine Epidemic Diarrhea Virus
PPE	Personal protective equipment
PRRA	Proline-Arginine-Arginine-Alanine
Q-assay	Quan assay
RdRp	RNA dependent RNA polymerase

RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RV	Rotavirus
S	Spike protein
SADS-CoV	Swine acute diarrhea syndrome coronavirus
SARS-rCoVs	SARS-related coronaviruses
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
TAE	Tris-acetate-EDTA
TGEV	Transmissible gastroenteritis virus
UNIMAS	Universiti Malaysia Sarawak
UNIMAS-AEC	UNIMAS-Animal Ethics Committee
UTR	Untranslated region
UV	Ultraviolet
V	Voltage
VNT	Virus neutralization test
VTM	Viral transport medium
W-assay	Watanabe assay
WGS	Whole-genome sequencing
WHO	World Health Organization
α -CoV	Alphacoronavirus
β -CoV	Betacoronavirus
δ -CoV	Deltacoronavirus
γ -CoV	Gammacoronavirus
omp	α -CoV membrane protein

°C Degree Celsius

μL Microlitre

CHAPTER 1

INTRODUCTION

1.1 Study Background

Emerging infectious diseases (EID) have become increasingly common in the 21st century, with approximately 75% originating from zoonotic sources (Jones et al., 2008; Slingenbergh et al., 2004; Tomori & Oluwayelu, 2023). Among these, bats are widely recognized as hosts for thousands of viruses belonging to at least 28 different families (Letko et al., 2020), some of which cause severe and often fatal diseases in both humans and animals. Notably, many of these viruses are only identified after triggering significant outbreaks in human or livestock populations (Hu et al., 2015). Research further highlights that bats harbor a remarkably higher number of viruses compared to other mammalian orders, underscoring their critical role as viral reservoirs (Olival et al., 2017).

One prominent group of viruses harbored by bats is the coronaviruses (CoVs), which are classified under the subfamily *Orthocoronavirinae* in the family *Coronaviridae*. These viruses are enveloped and are characterized as single-stranded, positive-sense RNA viruses. Notably, CoVs possess the largest continuous RNA genomes among mammalian viruses, with sizes ranging from 25,000 to 32,000 base pairs (Woo et al., 2009). Based on the International Committee on Taxonomy of Viruses (ICTV) classification, CoVs are divided into four genera: *Alphacoronavirus* (α -CoV), *Betacoronavirus* (β -CoV), *Gammacoronavirus* (γ -CoV), and *Deltacoronavirus* (δ -CoV) (Cui et al., 2019). This extensive genetic diversity within CoVs, combined with their ability to infect a wide range of hosts, highlights their potential to spill over into human populations, contributing to the emergence of novel infectious diseases (Bonavita et al., 2024; Cerri et al., 2023; De Klerk

et al., 2022; Forni et al., 2022; Guo et al., 2024; Olarte-Castillo et al., 2021; Su et al., 2016a; Vakulenko et al., 2021; Zhou et al., 2021).

Currently, seven human coronaviruses (HCoVs) have been identified, causing both respiratory and gastrointestinal illnesses. The earliest detections of HCoVs, HCoV-229E and HCoV-OC43, were reported in the 1960s and were associated with mild respiratory symptoms (Heikkinen & Järvinen, 2003). Later, HCoV-NL63 and HCoV-HKU1 were discovered in 2004 and 2005, respectively (Van Der Hoek et al., 2004; Woo et al., 2005). However, it was the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 that drew global attention to CoVs as serious public health threats. This virus infected approximately 8,500 individuals and had a case fatality rate of 11%, raising concerns about its pandemic potential (Chan-Yeung et al., 2003). Nearly a decade later, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012, causing severe respiratory distress and lung inflammation (Bermingham et al., 2012; Zaki et al., 2012). Most recently, SARS-CoV-2 was identified in Wuhan, China, in December 2019, following an outbreak of pneumonia cases, ultimately resulting in the COVID-19 pandemic.

These events have spurred growing interest in understanding human-animal interactions, particularly the role of bats as reservoirs for zoonotic viruses. Studies show that bats host a substantial diversity of CoVs, particularly α -CoVs and β -CoVs, which have been detected across frugivorous and insectivorous bat species in Asia, Africa, Europe, and the Americas (Anthony et al., 2013; Li et al., 2005; Mickleburgh et al., 2002; Raj et al., 2013; Tong et al., 2009). As the second-largest order of mammals, bats (order Chiroptera) occupy diverse ecological niches and exhibit varied diets, including insects, mammals, fish, blood,

nectar, fruit, and pollen (Teeling et al., 2005, 2018). Their ecological adaptability and ability to host multiple viruses make them a critical focus for studies on zoonotic spillover events.

Bats are predominantly nocturnal creatures, actively foraging at night and resting during the day. They inhabit a variety of natural shelters, including caves, trees, old buildings, and rock crevices. Beyond their ecological importance as pollinators and natural pest controllers (Baldwin et al., 2020; Fadini et al., 2018), bats have also attracted scientific interest due to their role as viral reservoirs. Many species use echolocation, emitting ultrasonic waves to navigate and hunt, which may facilitate the aerosolization and dispersal of pathogens, including viruses (Calisher et al., 2006; Springer et al., 2001). Their unique biological traits, such as long lifespans, migratory behaviors, and gregarious roosting patterns, further enhance their capacity to harbor and transmit viruses (Calisher et al., 2006; Serra-Cobo & López-Roig, 2016).

The emergence of SARS-CoV and the discovery of two other HCoVs such as HCoV-NL63 and HCoV-HKU1, further emphasize the importance of bats as reservoirs for zoonotic viruses. In less than 18 years, bat CoVs (BtCoVs) have made their third jump from animal to human. Since the first SARS-CoV epidemic in 2002/2003, bats have been identified as the natural reservoirs of SARS-CoV-like viruses (Li et al., 2005). These outbreaks have cemented the Coronaviridae as a family of zoonotic concern and stimulated a surge in viral discovery efforts in bats. These efforts appear to show that almost all HCoVs have zoonotic origins or otherwise circulate in animals, including human 229E (bats); MERS-CoV (camels); NL63 (bats) and OC43 (cattle). Even non-human CoVs such as porcine epidemic diarrhea virus (PEDV) may have emerged by host switching from other animals.

Overall, it seems that CoV diversity in bats is substantial that these viruses are prone to host switching (Woo et al., 2009) and that they are a current, historic, and future threat to public health (Drexler et al., 2014). Despite recent efforts, there are several notable gaps in our understanding of CoV diversity. In the case of SARS-CoV-2, a β -CoV, genetic studies have revealed striking similarities between the virus and other bat-borne β -CoVs, particularly those found in *Rhinolophus* species. Strains such as *Rhinolophus malayanus* RmYN02 (Zhou et al., 2020), *Rhinolophus affinis* RaTG13 (Zhou et al., 2020) , and more recent discoveries like *Rhinolophus acuminatus* RacCS203 (Wacharapluesadee et al., 2021) and *Rhinolophus malayanus* BANAL-52 (Temmam et al., 2022) highlight the evolutionary connections and zoonotic potential of BtCoVs.

Despite recent advancements, significant gaps remain in understanding CoV diversity and transmission dynamics, particularly in regions rich in bat biodiversity, such as Sarawak in Malaysian Borneo. Sarawak, home to 80 documented bat species across nine families (Pteropodidae, Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae, Hipposideridae, Vespertilionidae, Miniopteridae, and Molossidae) (Abdullah et al., 2021), offers an ideal setting for studying CoV diversity and evolution. Horseshoe bats (*Rhinolophidae*), believed to be ancestral reservoirs of epizoonotic CoVs, are endemic to this region and have been the focus of virological surveillance and bio-risk assessments. The closest known relatives to SARS-CoV-2, such as BANAL-52 (96.8% similarity) from *R. malayanus* and RaTG13 (96.1% similarity) from *R. affinis*) underscore the risks posed by bat-borne CoVs, with *R. affinis* also found in Sarawak, Malaysian Borneo, further emphasizing the urgency for focused research in this region.

Recent studies in Malaysia have revealed the presence of diverse CoVs in various bat species. These studies have been conducted in both Peninsular Malaysia and Malaysian Borneo, reflecting the widespread distribution of bats and their role as CoV reservoirs across the region. In Peninsular Malaysia, a study conducted in east coast Malaysia covering samples from three states, i.e. Terengganu, Kelantan and Pahang detected SARS-CoV-2-like viruses in 3% of sampled bats, showing 98-99% sequence identity to human SARS-CoV-2 (Gilbert et al., 2023). Another study done at the Krau Wildlife Reserve Forest, Pahang identified a bat β -CoV in a *Rhinolophus* spp. sample, which showed slight genetic differences compared to CoVs detected in other countries (Siew et al., 2023). Whereas, in Sarawak, Malaysian Borneo, both α -CoVs and β -CoVs were found in dusky fruit bats (*Pteropus lucasi*), with a 38.5% positivity rate (Habeebur-Rahman et al., 2023). Another study in Sarawak identified four novel CoV clades, including one closely related to *Sarbecovirus* (Tan et al., 2021). These findings highlight the importance of continued surveillance of BtCoVs in Malaysia, as they may pose potential public health concerns and contribute to our understanding of CoV diversity and evolution.

Early detection of novel CoVs is essential to prevent future zoonotic outbreaks. Accurate identification relies on molecular methods such as reverse transcription-polymerase chain reaction (RT-PCR) assays, which are highly sensitive and specific. Common approaches include the Quan (Q-assay) (Quan et al., 2010) and Watanabe (W-assay) (Watanabe et al., 2010) protocols, which target conserved regions of the RdRp gene. While these assays have proven effective in detecting CoVs across ecological settings (Anthony et al., 2017b; Corman et al., 2014; Hu et al., 2017; Wacharapluesadee et al., 2024), their application in Sarawak remains largely unexplored.

This study aims to detect CoVs carried by selected Bornean bats and evaluate the performance of the Q-assay and W-assay protocols in identifying CoVs across bat populations in the western and northern regions of Sarawak. By assessing the sensitivity and specificity of these molecular assays, this research seeks to enhance our understanding of CoV diversity, transmission dynamics, and potential spillover risks within this ecologically rich but understudied area. The findings will contribute to global surveillance efforts and strengthen pandemic preparedness strategies by enabling early detection of zoonotic threats.

The novelty of this study lies in its focus on Sarawak, a region of Malaysian Borneo where systematic BtCoV surveillance has been limited despite its exceptional biodiversity and extensive bat populations. Unlike previous studies that have largely concentrated on Peninsular Malaysia or neighboring Southeast Asian countries, this work provides baseline molecular data for Sarawak and applies a dual-assay approach to evaluate detection efficiency. By combining ecological insights with methodological evaluation, the study delivers both region-specific knowledge and a critical assessment of primer performance. Taking together, these contributions provide a unique foundation for advancing BtCoV surveillance in Borneo and beyond.

1.2 Problem Statement

The lack of comprehensive virological surveillance and genetic studies on bat-borne CoVs in Malaysia, particularly in Sarawak, presents significant challenges in understanding the zoonotic risks associated with Chiropterans. Despite the presence of species such as *R. affinis*, which are known to harbor CoVs closely related to SARS-CoV-2, limited information exists regarding the prevalence, genetic diversity, and evolutionary relationships

of these viruses within the region. This knowledge gap hinders effective risk assessment and preparedness for potential zoonotic transmission events.

Furthermore, diagnostic tools such as the Q-assay and W-assay protocols, which are commonly used for detecting BtCoVs, have yet to be thoroughly evaluated in this context, raising concerns about their sensitivity and specificity. Without a clear understanding of the diagnostic efficacy of these assays, accurate detection and monitoring of bat-borne CoVs remain constrained. Additionally, the absence of a centralized and updated database linking Chiropteran species to their associated CoVs further complicates efforts to monitor and mitigate the risk of spillover events. Limited screening at the human-animal interface exacerbates these challenges, particularly in regions where interactions between bats and humans are frequent.

1.3 Objectives

The study aims:

- i. To investigate the presence of BtCoVs in Chiropterans in Sarawak, Malaysia
- ii. To update the list of Chiropterans with associated BtCoVs and build a new database in Sarawak, Malaysia.
- iii. To investigate the molecular phylogeny and genetic diversity between CoVs Sarawak, Malaysia with CoVs found in the other part of the world using two different PCR pan-CoV assays, Q-assay and W-assay.
- iv. To determine the sensitivity and specificity of the Q-assay and W-assay.

1.4 Research Questions

The research questions are:

- i. Are BtCoVs present in Chiropterans in Sarawak, Malaysia?
- ii. Which Chiropterans species in Sarawak, Malaysia are associated with BtCoVs, and how can a comprehensive database of these host-associations be established?
- iii. How do the molecular phylogeny and genetic diversity of CoVs in Sarawak Chiropterans compare with CoVs found in other parts of the world when analyzed using two pan-CoV PCR assays (Q-assay and W-assay)?
- iv. What are the sensitivity and specificity of the Q-assay and W-assay in detecting BtCoVs in Sarawak, Malaysia?

1.5 Research Hypothesis

H01: There is no significant presence of BtCoVs in Chiropterans across various regions of Sarawak, Malaysia.

HA1: BtCoVs are present in Chiropterans across various regions of Sarawak, Malaysia, with prevalence rates varying by species and location.

H02: There is no significant association between Chiropteran species in Sarawak, Malaysia and the presence of BtCoVs, and therefore a species-virus database cannot be developed.

HA2: There is a significant association between Chiropteran species in Sarawak, Malaysia and the presence of BtCoVs, and therefore a species-virus database cannot be developed.

H03: There is no significant phylogenetic or genetic diversity between CoVs detected in Malaysian Chiropterans and those reported from other parts of the world.

HA3: There is significant phylogenetic or genetic diversity between CoVs detected in Malaysian Chiropterans and those reported from other parts of the world.

H04: There is no significant difference in the sensitivity and specificity of the Q-assay and W-assay PCR methods in detecting BtCoVs.

HA4: There is a significant difference in the sensitivity and specificity of the Q-assay and W-assay PCR methods, with one assay showing higher performance in detecting BtCoVs.

1.6 Chapter Summary

There are six chapters included in this thesis. Chapter 1 is the general introduction and objectives of this study. Chapter 2 presents the literature review for this study based on the previous works which have been done related to the current study. Chapter 3 discusses the materials and methods used to conduct the research, including sample collection, molecular techniques, and statistical analyses. Chapter 4 presents the results of the study, including the detection of BtCoVs, phylogenetic analysis, and evaluation of the PCR assays. Chapter 5 provides a detailed discussion of the findings, comparing them with previous research and highlighting the implications for public health and zoonotic risk. Finally, Chapter 6 concludes the thesis with a summary of the key findings, followed by recommendations for future research and potential public health actions.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural History of Bats

Bats are unique among placental mammals as the only group capable of sustained flight, with their distribution spanning the globe except for Antarctica, the Arctic (though a few species reach the Arctic Circle), and certain remote oceanic islands. Of the 1,314 genera and 6,495 species of placental mammals, bats form the second largest mammalian group after rodents (Glover & Altringham, 2008). With over 1,400 species of bats, they account for nearly a one-fifth of mammals in the world (Ismaili et al., 2023). Bats' unique flight capabilities have influenced their diversification, life history, and geographic distribution across multiple spatial scales (Peixoto et al., 2018).

Bats belong to the order Chiroptera, a name derived from the Greek words *cheir* (hand) and *pteron* (wing). One key characteristic that distinguishes these groups is echolocation, a process used by many bats for navigation and hunting. Echolocation ability plays a significant role in defining phylogenetic groups within the Chiroptera order. Traditionally, Chiroptera was divided into two suborders: Microchiroptera (echolocating via the larynx) and Megachiroptera (the Pteropodidae, which do not echolocate through the larynx) (Jones & Teeling, 2006). The Microchiroptera were further split into Yinpterochiroptera (those with moveable or absent premaxillaries) and Yangochiroptera (those with fused premaxillaries). However, the Microchiroptera group has been found to be paraphyletic, as molecular studies suggest that some members, such as the Rhinolophoidea, are more closely related to the Pteropodidae than to other microbats (Springer et al., 2001). This has led to a reclassification of bats into two major groups: Yinpterochiroptera, which

includes both Pteropodidae and Rhinolophoidea, and Yangochiroptera, which consists of the remaining Microchiroptera excluding the Rhinolophoidea. This new taxonomy better reflects the evolutionary relationships among bat species (Figure 2.1).

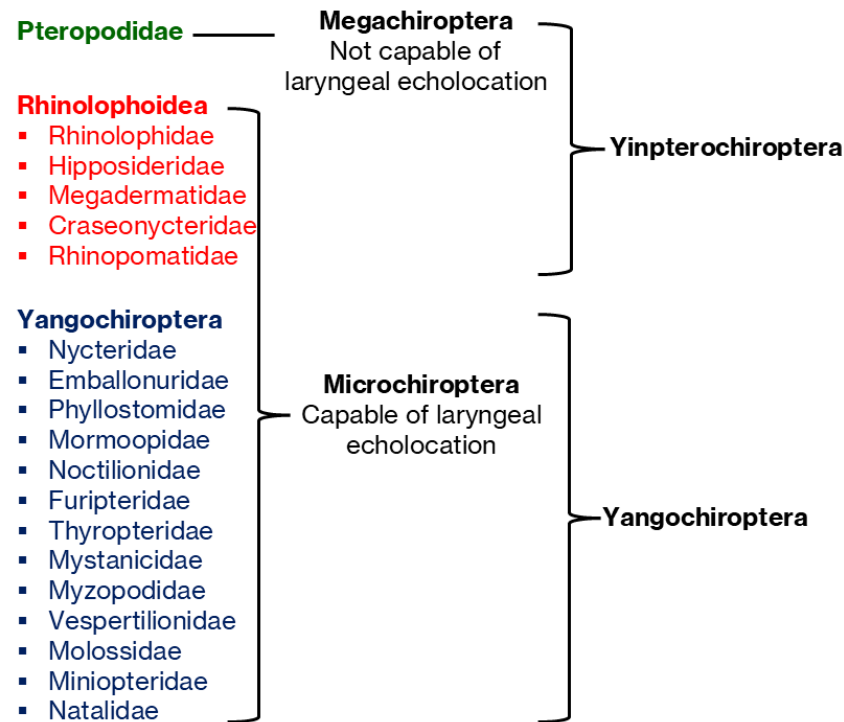


Figure 2.1: Phylogenetic groupings of chiropterans, highlighting the subdivisions of Chiroptera into Megachiroptera/Microchiroptera and Yinpterochiroptera/Yangochiroptera. Adapted from Anderson & Ruxton (2020)

Bats exhibit a wide distribution, highlighting their evolutionary success among mammals. The Vespertilionidae family boasts the largest number of species (over 500) and inhabits nearly all bat-populated regions (Gunnell et al., 2017). Molossidae and Emballonuridae families are found on every continent except Antarctica and the Arctic. However, some bat families have restricted distributions: Craseonycteridae (some members found only in Thailand), Myzopodidae (Africa: Madagascar and Egypt), Mystacinidae (Oceania), and Noctilionoidea (America) (Gunnell et al., 2014; Russell et al., 2008). Bats

also vary greatly in size and weight across species. The smallest bat, Kitti's hog-nosed bat (*Craseonycteris thonglongyai*), also known as the bumblebee bat and considered the smallest mammal, weighs as little as two grams found in parts of Thailand and Myanmar. (Pereira et al., 2006). In contrast, megabats, which belong to the single family *Pteropodidae* and are commonly known as "Old World fruit bats," can weigh over one kilogram, with species like flying foxes (*Pteropus* spp.) achieving wingspans of nearly two meters (Bonaccorso, 1998; Fenton & Simmons, 2015).

Many bat species show habitat preferences ranging from forests and pasturelands to urban settings such as houses and city buildings (Russo & Ancillotto, 2014). In urban areas, some bat species show resilience and adaptability, exploiting forest fragments or roosting directly in buildings (Betke et al., 2024). Urban habitats with fruiting trees and streetlamps attract insects which serve as food sources for insectivorous bats (Nunes et al., 2016). Although urbanization can negatively affect some species, bats overall are ecologically flexible and can persist in a variety of anthropogenic landscapes (Lehrer et al., 2021; Russo & Ancillotto, 2014). Bats also inhabit savannahs and deserts. Specific studies have documented bat roosting patterns and activity in neotropical savannas and other dry habitats (Aguirre et al., 2003). Their occupation of diverse trophic niches from nectar feeding to insectivory and carnivory allows them to utilize a broad spectrum of environmental conditions (Klarner et al., 2017; Ramos Pereira & Palmeirim, 2013).

Bat species have adapted themselves to feed on different food resources such as arthropods, fruit, nectar, pollen, small mammals, fish, frogs and blood (Teeling et al., 2018b). They are commonly categorized into six main trophic groups: frugivores, nectarivores, insectivores, carnivores, piscivores, and sanguivores, which helps to define

their ecological roles. The ability to echolocate and fly allows them to find a niche to navigate and hunt in darkness. As insectivores, they contribute to natural pest control, while some species act as pollinators and seed dispersers, promoting forest regeneration and plant diversity (Kasso & Balakrishnan, 2013). However, bat populations face numerous threats, including habitat destruction, pollution, and climate change. Conservation efforts are essential to preserve bat populations and their habitats, emphasizing the need for habitat preservation, public awareness campaigns, and legal protection (Ismaili et al., 2023; Kasso & Balakrishnan, 2013).

Roosting behavior is another fundamental aspect of bat natural history, exhibiting substantial variation across species (Boerma & Swartz, 2024). Many bats, such as *Rhinolophus* and *Miniopterus* species, are cave-dwellers forming large colonies sometimes numbering in the thousands or millions (Mohd-Kanapiah et al., 2025). Others, like *Cynopterus brachyotis*, demonstrate flexibility by roosting in foliage, hollow trees, or human structures. The selection of roosting sites is influenced by microclimatic conditions including temperature, humidity, and protection from predators, which shape social organization and population densities (Aguillon et al., 2023; Betke et al., 2024). This variation in roosting has important implications for pathogen dynamics. Dense colonies in caves may facilitate viral persistence and transmission, while species using more varied roosts, including anthropogenic ones, may increase opportunities for human-bat contact (Calisher et al., 2006; Dutheil et al., 2021). Approximately 40% of bat species are known to roost in anthropogenic structures, underlining extensive adaptability in roosting ecology (Betke et al., 2024).

2.2 Bats as Reservoir Hosts for Viruses

Bats with their diverse ecology, biology, and unique traits make them ideal viral reservoirs. Approximately 75% of EIDs in humans are zoonotic, with many linked to wildlife hosts such as bats (Allen et al., 2017). An intermediate host is often required for transmission of bat borne viruses to human (Field et al., 2001; Halpin et al., 2011; Wang & Anderson, 2019). For instance, the outbreaks of SARS-CoV and MERS-CoV were caused by viruses originated from bats, but were transmitted to humans through an intermediate host, that is civets and dromedary respectively (Fan et al., 2019; Hu et al., 2015; Li et al., 2005; Zaki et al., 2012). Bats are natural reservoirs for numerous viruses, both DNA and RNA, including zoonotic viruses like Ebola, Marburg, rabies, and Hendra virus (Fan et al., 2019; Hayman, 2016; Kohl & Kurth, 2014; Li et al., 2005; Megali et al., 2010; Smith & Wang, 2013; Wang & Anderson, 2019).

DNA viruses represent a smaller proportion of bat-associated viruses (Figure 2.2). The most prevalent DNA viruses in bats are herpesviruses, circoviruses, and parvoviruses (Ge et al., 2011; James et al., 2020; Vidovszky et al., 2023; Wu et al., 2016). Circoviruses are predominantly found in Rhinolophidae and Vespertilionidae bats in Asia and Africa and demonstrate high genetic diversity due to mutation and recombination (Ge et al., 2011; Zhu et al., 2018). The recently emerged porcine circovirus type 3, suspected to have originated in bats, exemplifies the potential for cross-species transmission (Li et al., 2018). Bat polyomaviruses also display extensive diversity, possibly driven by intra- and interspecies transmission within densely populated roosting colonies (Paskey et al., 2020; Tan et al., 2020).

RNA viruses make up most bat-associated viruses and are more prone to cross-species transmission with higher pathogenicity (Cui et al., 2019; Eaton et al., 2006; Woo & Lau, 2019; Zhou et al., 2021). Common RNA viruses in bats include coronaviruses, rhabdoviruses, paramyxoviruses, and astroviruses, followed by filoviruses, picobirnaviruses, caliciviruses, reoviruses, and flaviviruses. Viral distribution and composition vary across bat populations and habitats (Dutheil et al., 2021). While rhabdoviruses are prevalent in the Americas and Europe, paramyxoviruses are common in Oceania, and coronaviruses dominate in Asia and Africa (Lorenzo et al., 2021; Paskey et al., 2020). It is important to note that current data may reflect sampling bias due to limited research on other viral types in certain regions (Young & Olival, 2016).

Approximately 80% of identified bat-associated RNA viruses are found in just three bat families: *Vespertilionidae*, *Rhinolophidae*, and *Pteropodidae*. The *Rhinolophus* bats (family *Rhinolophidae*) are natural reservoirs of SARS-like CoVs (Belouzard et al., 2012; Ge et al., 2013; Lau et al., 2005; Li et al., 2005; Rihtarič et al., 2010). These bats are insectivores with a wide distribution across Southeast Asia, Africa, eastern Oceania, and Western Europe. MERS-CoV is associated with bats from *Vespertilionidae* (the most diverse and widespread bat family) and *Nycteridae* (Cui et al., 2019; de Groot et al., 2013). Henipaviruses and other paramyxoviruses are often found in *Pteropodidae* (fruit bats), which have a broad distribution and long-distance flight capabilities. Filoviruses (like Marburg and Ebola) are also predominantly found in *Pteropodidae*. Additionally, *Pteropodidae* bats can harbor zoonotic viruses such as rotaviruses (RVs), which cause diarrheal disease in various species. Bat RVs show significant genetic diversity and may be the source of several human and animal rotaviruses (Paskey et al., 2020; Simsek et al., 2021).

While some bat families, like *Myzopodidae*, have not yet been found to carry viruses, this may be due to limited research (Szentivanyi et al., 2023).

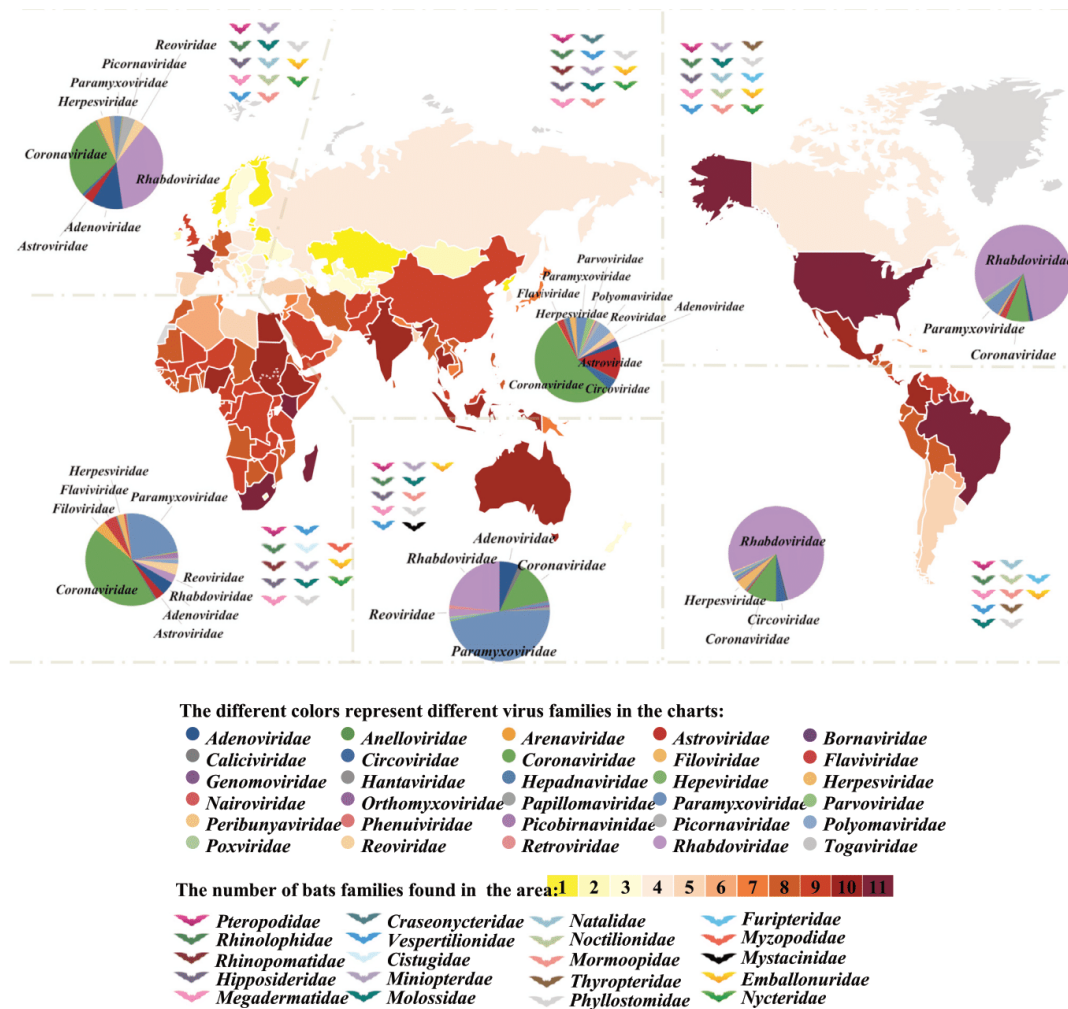


Figure 2.2: Global distribution of bats and their viruses. Bat pictograms represent bat families present on each continent, with color intensity (yellow to red) indicating the number of bat families in each country. The pie charts show the percentage of bat-associated viruses from each family identified in the respective continents, based on publicly available sequence data from the Bat-Associated Virus database (<http://www.mgc.ac.cn/DBatVir/>) as of July 16, 2020, and GenBank data from July 16, 2020, to August 25, 2020 (Tian et al., 2022)

Bats have unique characteristics in biological, physiological and immunology that make them - the ideal candidates to host and spread viruses. First, they are the only mammals capable of powered flight as some species can migrate several hundred miles for winter hibernation which leads to viral spread over long distances (Calisher et al., 2006). Secondly, the longevity, for instance species *Myotis brandtii* can live up to 41 years, also facilitates long virus persistence (Podlutsky et al., 2005; Smith & Wang, 2013; Wilkinson & Adams, 2019; Wilkinson & South, 2002). Third, bats roost in colonies up to a million individuals per colony, which supports virus transmission between themselves (Calisher et al., 2006).

Bats are phylogenetically the oldest mammals on earth, their immune system was able to adapt and survive viral infections over a long time of co-evolution (Apoorva & Singh, 2024; Gupta et al., 2021). They appear to control viral replication more effectively than other mammals (Baker et al., 2013; Zhang et al., 2013). Their robust immune systems, including genes under positive selection, often result in asymptomatic infections (Das et al., 2025; Morales et al., 2025; Pei et al., 2024). Certain bat species are capable of harboring multiple pathogens without showing signs of illness, owing to their specialized immune system adaptations (Sia et al., 2022). However, even without symptoms, bats shed viruses through various bodily fluids (Ruiz-Aravena et al., 2021). Transmission between bats occurs through the urine-oral route, while other mammals can be infected through contaminated food or fomites (Arankalle et al., 2011; Edson et al., 2015; Islam et al., 2016; Nyakarahuka et al., 2019). Even their echolocation, using ultrasonic waves emitted through the mouth or nose, may contribute to viral aerosolization (Calisher et al., 2006). These high-frequency vocalizations can generate droplets or aerosols of saliva, mucus, or oropharyngeal fluids, enabling virus transmission among closely roosting individuals. Supporting evidence includes the detection of rabies virus in mucus of naturally infected Mexican free-tailed bats,

suggesting viruses may be expelled during echolocation. Such aerosolization could facilitate both intra-species transmission within bat colonies and inter-species transmission when bats interact with other animals or humans (Calisher et al., 2006).

They are not only ideal hosts and reservoirs for various types of viruses, but several factors also contribute to the efficient transmission of viruses. These include intrinsic factors like body condition, sex, social and reproductive status as well as extrinsic factors affecting the habitat of the bats such as cave destruction, logging, drought, and bush fires (Calisher et al., 2006; Castro & Michalski, 2014; Meyer et al., 2015; Plowright et al., 2008; Smith & Wang, 2013; Wimsatt & Villa-R, 1970). The bats are often forced to move closer to domestic areas due to habitat loss and lack of food, hence increasing the risk of virus transmission to other species including humans and farm animals (Blakey et al., 2019; Brearley et al., 2013; Field et al., 2016; Smith & Wang, 2013).

2.3 Family Coronaviridae

Coronaviridae are enveloped, positive-strand RNA viruses infecting various animals. They belong to the order Nidovirales (Andersen et al., 2020; Drosten et al., 2003; Fan et al., 2019; Fehr & Perlman, 2015), which are enveloped, positive-strand RNA viruses within the suborder Cornidovirineae (Figure 2.3). Phylogenetic studies suggest Nidovirales, along with Picornavirales, Caliciviridae, and Astroviridae, form a distinct RNA virus supergroup (Koonin et al., 2020; Wolf et al., 2018). Nidovirales infect diverse animal hosts, implying horizontal virus transfer across species (Dolja & Koonin, 2018). Coronaviridae are divided into two subfamilies: Letovirinae and Orthocoronavirinae (Adams et al., 2017; Siddell et al., 2020; Walker et al., 2019). Orthocoronavirinae are divided into four genera and include seven species/subspecies infecting humans: two α -CoVs (HCoV-229E and HCoV-NL63)

and five β -CoVs (HCoV-OC43, HKU1, MERS-CoV, SARS-CoV, and SARS-CoV-2) (Andersen et al., 2020; Drosten et al., 2003; Fan et al., 2019; Fehr & Perlman, 2015).

2.3.1 Subfamily Orthocoronavirinae - Classification

Based on genome organization and phylogenetic relationships by International Committee on Taxonomy of Viruses (ICTV), CoVs have been classified into the subfamily Orthocoronavirinae that consists of four genera α -CoV, β -CoV, δ -CoV, and γ -CoV. The α -CoV and β -CoV infect only mammals. Whereas the δ -CoV and γ -CoV are normally excluded from bat CoV studies because their primary hosts are birds, with only occasional spillovers to mammals (Mavrodiev et al., 2020; Woo et al., 2012). This thesis focuses on α - and β -CoVs, as these lineages have extensively co-evolved with bat hosts and remain central to bat-related CoV research.

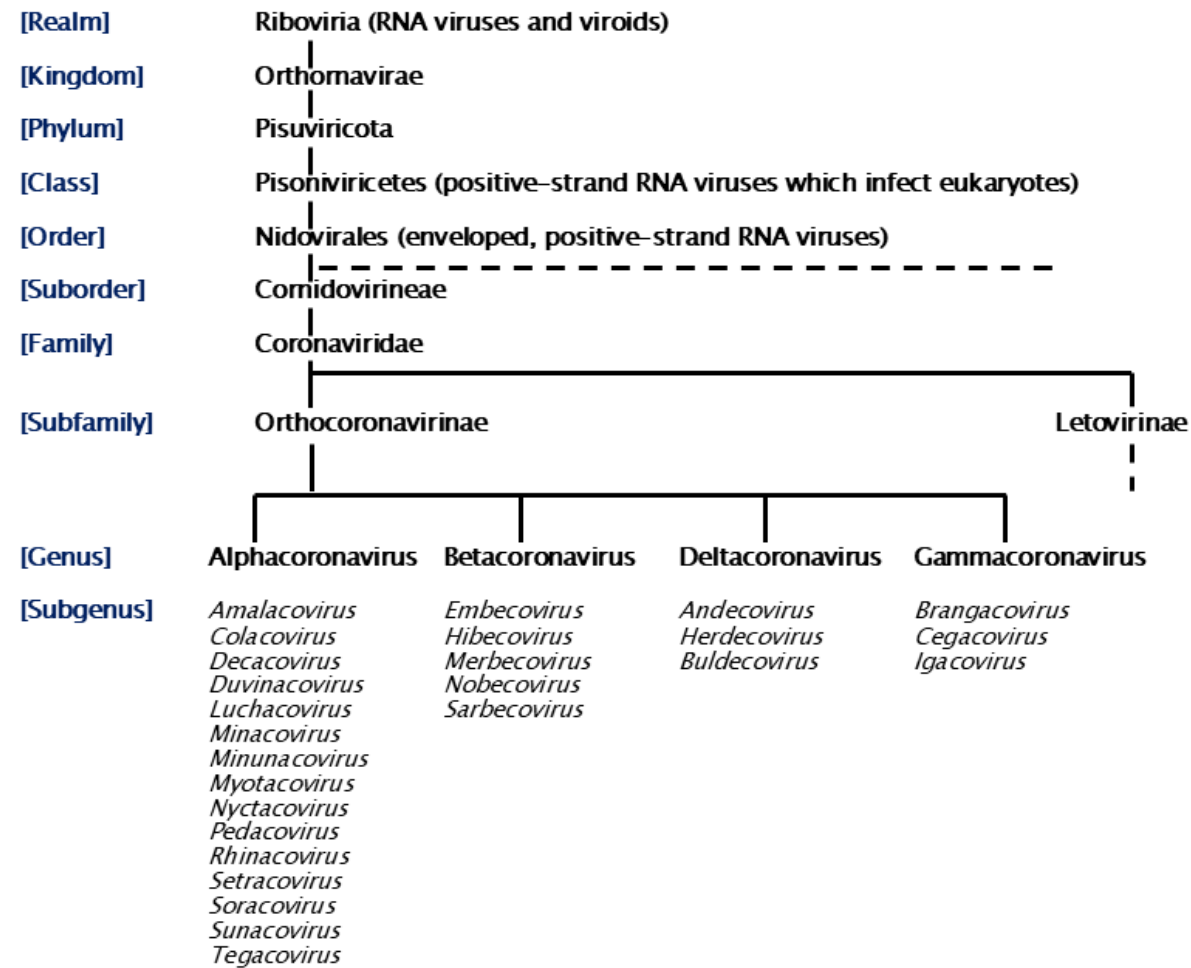


Figure 2.3: The Nidovirales taxonomy. Taxonomic levels are denoted in square brackets. Adapted from Zmasek et al. (2022)

2.3.1.1 Alphacoronavirus (α -CoV)

The α -CoVs are a monophyletic group within the *Orthocoronavirinae* subfamily, set apart by their unique non-structural protein 1 (nsp1). This nsp1 differs in both size and amino acid sequence from the nsp1 of β -CoVs and is not found in δ -CoVs or γ -CoVs (Connor & Roper, 2007). Additionally, the accessory gene ORF3 is present in most α -CoV species (Figure 2.4). This gene encodes a non-essential, multi-spanning α -CoV membrane protein (*amp*) specific to α -CoVs (Müller et al., 2010; Vlasova et al., 2011). In some α -CoVs, *amp* is the only accessory protein; however, other α -CoV genomes, like *Alphacoronavirus 1*, encode up to six accessory genes.

Based on ICTV classification, genus α -CoV comprises of 1 subgenera: *Amalacovirus* (monotypic) *Colacovirus* (monotypic), *Decacovirus* (four species), *Duvinacovirus* (two species), *Luchacovirus* (monotypic), *Minacovirus* (monotypic), *Minunacovirus* (two species), *Myotacovirus* (monotypic), *Nyctacovirus* (four species), *Pedacovirus* (four species), *Rhinacovirus* (monotypic), *Setracovirus* (two species), *Soracovirus* (monotypic), *Sunacovirus* (monotypic), and *Tegacovirus* (monotypic).

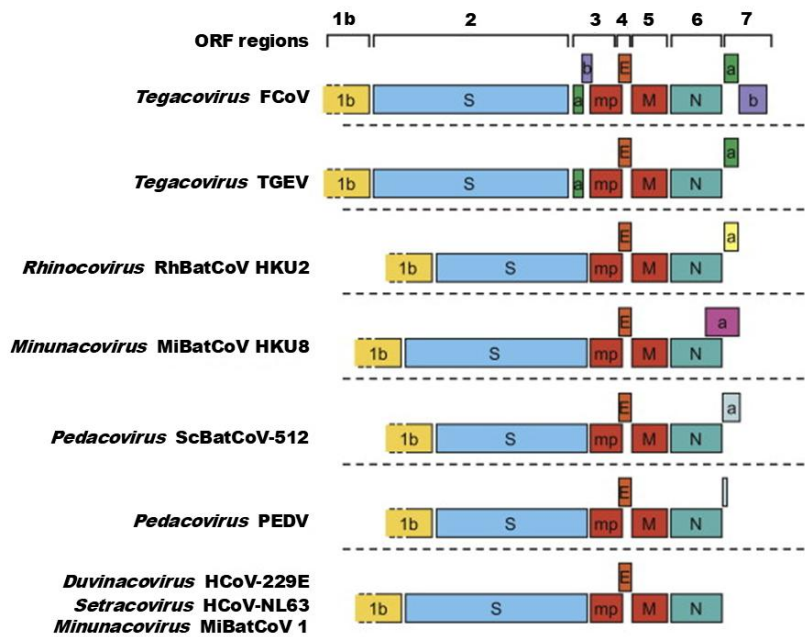


Figure 2.4: Genome organization of representative α -CoVs. The schematic shows the arrangement of open reading frames (ORFs) in selected α -CoVs subgenera. The ORF1b (yellow) encodes non-structural proteins involved in replication, followed by structural proteins including spike (S, blue), envelope (E, orange), membrane (M, red), and nucleocapsid (N, cyan). Additional accessory proteins (mp, a, b) are shown in various colors and vary among different α -CoV species. This comparison highlights the conserved core genome structure with variations in accessory gene content among FCoV, TGEV, RhBatCoV HKU2, MiBatCoV HKU8, ScBatCoV-512, and HCoVs such as HCoV-229E and HCoV-NL63. Adapted from (Coronaviridae, 2012)

The α -CoVs have a broad mammalian host range. Bats are infected by members of the subgenera *Colacovirus*, *Decacovirus*, *Duvinacovirus*, *Minuacovirus*, *Myotacovirus*, *Nyctacovirus*, *Pedacovirus*, *Rhinacovirus*, and *Setracovirus*. Pigs experience enteric diseases caused by *Pedacovirus* and *Rhinacovirus* (Lau et al., 2007; Pensaert & de Bouck, 1978; Zhou et al., 2018a). Humans are infected by two α -CoVs: HCoV-229E (*Duvinacovirus*) and HCoV-NL63 (*Setracovirus*) (Farsani et al., 2012; Tyrrell et al., 1965; Van Der Hoek et al., 2004). Other α -CoVs include Lucheng Rn rat CoV (*Luchacovirus*) in brown rats (Wang et al., 2015), mink CoV (*Minacovirus*) in American mink (Vlasova et al., 2011), shrew CoV Tibet-2014 (*Soracovirus*) in common shrews (Wu et al., 2018), *Suncus*

murinus CoV Xingguo-74 (*Sunacovirus*) in Asian house shrews, and *Alphacoronavirus 1* (*Tegacovirus*) infecting dogs, cats, and pigs (Whittaker et al., 2018). The detailed information on the member species of α -CoVs is presented in Table 2.1.

Table 2.1: Member Species of α -CoVs Classified by the ICTV According to the Most Recent Taxonomy. The Table Lists the Genus, Subgenus, Virus Name, Representative Isolate, GenBank Accession Number, Available Sequence, and Abbreviation

Genus	Subgenus	Species	Virus Name	Isolate	Accession Number	Available Sequence	Abbrev.
α -CoV	<i>Amalacovirus</i>	<i>Alphacoronavirus almalfi</i>	Bat alphacoronavirus isolate AMA_L_F	AMA_L_F	MT663548	Complete genome	BtCoV-AMA-L-F
	<i>Colacovirus</i>	<i>Alphacoronavirus myotis</i>	Bat coronavirus CDPHE15	bat/USA/CDPH E15/2006	KF430219	Complete genome	BtCoV CDPHE15
	<i>Decacovirus</i>	<i>Alphacoronavirus australiense</i>	Alphacoronavirus sp. WA3607	WA3607	MK472070	Complete genome	ACoV-WA3607
	<i>Decacovirus</i>	<i>Alphacoronavirus ferrumequini</i>	BtRf-AlphaCoV/HuB2013	BtRf-HuB2013	KJ473807	Complete genome	BtRf-AlphaCoV
	<i>Decacovirus</i>	<i>Alphacoronavirus hipposideri</i>	Hipposideros pomona bat coronavirus CHB25	CHB0025	MN611525	Complete genome	HipPBCoV-CHB25

Table 2.1 continued

	<i>Decacovirus</i>	<i>Alphacoronavirus roussetti</i>	Rousettus bat coronavirus HKU10	183A	JQ989270	Complete genome	BtCoV HKU10
	<i>Duvinacovirus</i>	<i>Alphacoronavirus chicaoense</i>	Human coronavirus 229E	229E	AF304460	Complete genome	HCoV_229E
	<i>Duvinacovirus</i>	<i>Alphacoronavirus chicaoense</i>	Human coronavirus 229E	J0304	JX503061	Complete genome	HCoV_229E
	<i>Luchacovirus</i>	<i>Alphacoronavirus ratti</i>	Lucheng Rn rat coronavirus	Lucheng-19	KF294380	Complete genome	LRNV
	<i>Minacovirus</i>	<i>Alphacoronavirus neovisontis</i>	Mink coronavirus	WD1127	HM245925	Complete genome	MCoV
	<i>Minunacovirus</i>	<i>Alphacoronavirus miniopteri</i>	Miniopterus bat coronavirus HKU8	AFCD77	EU420139	Complete genome	Mi-BatCoV_HKU8

Table 2.1 continued

	<i>Minunacovirus</i>	<i>Alphacoronavirus pusilli</i>	Miniopterus bat coronavirus 1	AFCD62	EU420138	Complete genome	Mi-BatCoV_1A
	<i>Myotacovirus</i>	<i>Alphacoronavirus ricketti</i>	BtMr-AlphaCoV/SAX2011	BtMr-SAX2011	KJ473806	Complete genome	BtMr-AlphaCoV
	<i>Nyctacovirus</i>	<i>Alphacoronavirus chalinolobi</i>	Alphacoronavirus sp. WA2028	WA2028	MK472068	Complete genome	ACoV-WA2028
	<i>Nyctacovirus</i>	<i>Alphacoronavirus nyctali</i>	BtNv-AlphaCoV/SC2013	BtNv-SC2013	KJ473809	Complete genome	BtNv-AlphaCoV
	<i>Nyctacovirus</i>	<i>Alphacoronavirus pipistrelli</i>	Alphacoronavirus bat coronavirus	P.kuhlii/Italy/3398/2015	MH938449	Complete genome	PK-BatCoV
	<i>Nyctacovirus</i>	<i>Alphacoronavirus tylonycteridis</i>	Tylonycteris bat coronavirus HKU33	GZ151867	MK720944	Complete genome	TyBCoV-HKU33

Table 2.1 continued

	<i>Pedacovirus</i>	<i>Alphacoronavirus finnoniense</i>	Bat alphacoronavirus BtCoV/020_16/M.dau/FIN/2016	020_16	MG923574	Coding- complete genome	BtCoV/020_16/ M.dau/FIN/2016
	<i>Pedacovirus</i>	<i>Alphacoronavirus gouldii</i>	Alphacoronavirus sp. WA1087	WA1087	MK472067	Complete genome	ACoV-WA1087
	<i>Pedacovirus</i>	<i>Alphacoronavirus porci</i>	Porcine epidemic diarrhea virus	CV777	AF353511	Complete genome	PEDV
	<i>Pedacovirus</i>	<i>Alphacoronavirus scotophili</i>	Scotophilus bat coronavirus 512	512/2005	DQ648858	Complete genome	Sc-BatCoV_512
	<i>Rhinacovirus</i>	<i>Alphacoronavirus rhinolophi</i>	Rhinolophus bat coronavirus HKU2	GD/430/2006	EF203064	Complete genome	Rh- BatCoV_HKU2
	<i>Setracovirus</i>	<i>Alphacoronavirus amsterdamense</i>	human coronavirus NL63	Amsterdam I	AY567487	Complete genome	HCoV_NL63

Table 2.1 continued

	<i>Setracovirus</i>	<i>Alphacoronavirus triaenopis</i>	NL63-related bat coronavirus	BtKYNL63-9b	KY073745	Complete genome	BtKYNL63
	<i>Soracovirus</i>	<i>Alphacoronavirus soricis</i>	Common shrew coronavirus Tibet-2014	Shrew-CoV/Tibet2014	KY370053	Complete genome	Sa-CoV_T14
	<i>Sunacovirus</i>	<i>Alphacoronavirus sunci</i>	Suncus murinus coronavirus Xingguo-74	Xingguo-74	KY967715	Coding-complete genome	Sm-CoV_X74
	<i>Tegacovirus</i>	<i>Alphacoronavirus suis</i>	Transmissible gastroenteritis virus	PUR46-MAD	AJ271965	Complete genome	TGEV

2.3.1.2 Betacoronavirus (β -CoV)

The β -CoVs are a monophyletic group within the *Orthocoronavirinae* subfamily, distinguished by unique nsp1 characteristics not found in other CoV groups. They infect a wide range of mammals, and some, like SARS-CoV, SARS-CoV-2, and MERS-CoV, pose significant threats to human health due to their high transmissibility and/or fatality rates (Drosten et al., 2003; Ksiazek et al., 2003; Zaki et al., 2012; Zhu et al., 2020). The presence of various accessory genes further contributes to the diversity within this group. Like α -CoVs, β -CoV infect diverse mammalian hosts. The genus β -CoV consists of five subgenera: *Embecovirus* (five species), *Hibecovirus* (monotypic), *Merbecovirus* (four species), *Nobecovirus* (three species) and *Sarbecovirus* (four species).

Within the *Embecoviruses* subgenus, several viruses and their hosts have been identified: *Betacoronavirus 1* and China rattus CoV HKU24 have been found in rats, Murine CoV infects mice, Myodes CoV 2JL14 infects rodents, and H-CoV HKU1 infects humans (Woo et al., 2005; Wu et al., 2018). Notably, *Betacoronavirus 1* exhibits cross-species transmission, having been identified in various mammals, including sable antelopes (*Hippotragus niger*), cattle (*Bos taurus*), waterbuck (*Kobus ellipsiprymnus*), dog (*Canis lupus familiaris*), white-tailed deer (*Odocoileus virginianus*), dromedary camel (*Camelus dromedarius*), pig (*Sus scrofa domesticus*), and human (Bakkers et al., 2017; Woo et al., 2014). The absence of *Embecoviruses* in bats suggests a possible rodent origin for this subgenus (Zhou et al., 2021).

Bats are the sole hosts for *Hibecovirus* and *Nobecovirus* (Zhou et al., 2021). *Merbecoviruses*, including MERS-CoV (the cause of the MERS-CoV epidemic), have been found in bats, hedgehogs, camels, and humans (de Groot et al., 2013; Lau et al., 2018; Stalin

Raj et al., 2014; Woo et al., 2007; Zaki et al., 2012). The subgenus *Sarbecoviruses* include SARS-CoV and SARS-CoV-2, the causative agents of the 2003 SARS epidemic and the COVID-19 pandemic, respectively (Drosten et al., 2003; Ksiazek et al., 2003; P. Zhou et al., 2020). SARS-CoV infects humans, bats, and palm civets, whereas SARS-CoV-2 has a broader host range, including bats, pangolins, various felines, ferrets, otters, gorillas, dogs, mice, hamsters, rabbits, and mink (Lau et al., 2005; Li et al., 2005; Nyberg et al., 2022). While most of these are considered dead-end hosts, bidirectional transmission has been observed between mink/hamsters and humans ((Wong et al., 2021; Yen et al., 2022).

In term of the genome organization and replication (Figure 2.5), the *Embecovirus* subgenus of β -CoVs is unique due to the presence of a haemagglutinin-esterase (HE) gene situated between ORF1 and the S gene (Zhou et al., 2021)). This gene is thought to have arisen from heterologous recombination between an ancestral *Embecovirus* and an influenza C virus (Zmasek et al., 2022). *Hibecovirus*, represented by the single species Bat Hp-betacoronavirus Zhejiang2013 (found in *Hipposideros pratti* bats), has a unique ORF2 positioned downstream of ORF1 and upstream of the S gene (Wu et al., 2016). Further molecular characterization of this virus is needed (Zhou et al., 2021). The detailed information on the member species of α -CoVs is presented in Table 2.2.

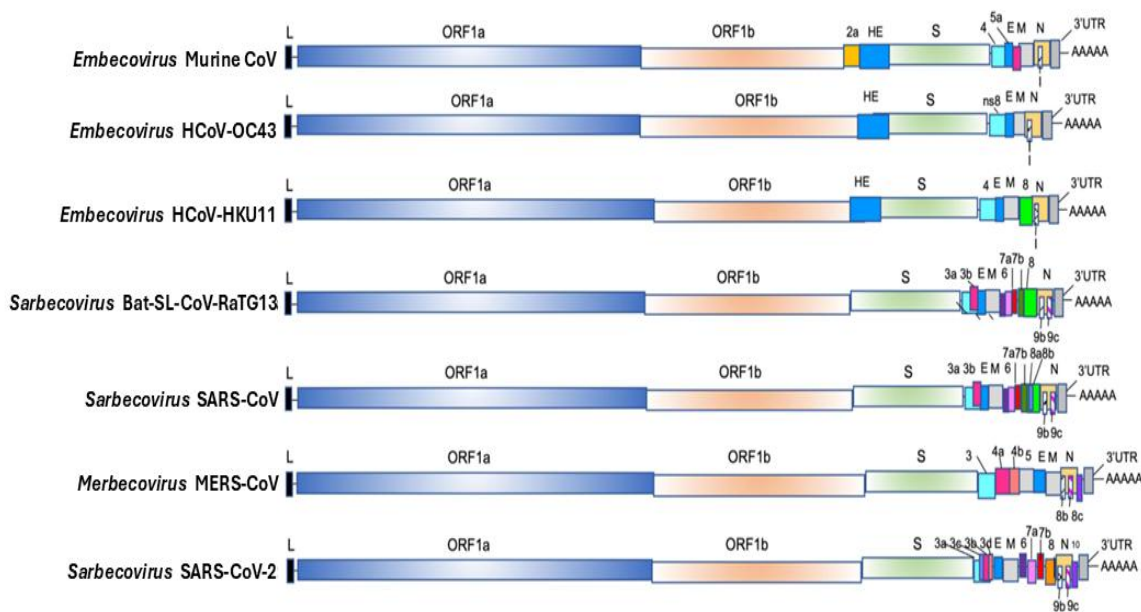


Figure 2.5: Genomic organization of representative β -CoVs from the subgenera *Embecovirus*, *Sarbecovirus*, and *Merbecovirus*, showing the conserved gene order from 5' to 3'. The 5'-terminal two-thirds of the genome encode two overlapping polyproteins, pp1a and pp1b, which are cleaved into 16 nonstructural proteins (nsps) responsible for genome replication and transcription. The 3'-end of the genome encodes four major structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Species-specific accessory genes are interspersed throughout the genome or embedded as alternative open reading frames (ORFs) within structural genes. Notably, SARS-CoV (GenBank: AY274119) contains 9 accessory ORFs (3a, 3b, 6, 7a, 7b, 8a, 8b, 9b, 9c), MERS-CoV (GenBank: NC_019843) retains 6 ORFs (3, 4a, 4b, 5, 8b, 8c), and SARS-CoV-2 (GenBank: NC_045512) has 11 ORFs (3a, 3b, 3c, 3d, 6, 7a, 7b, 8, 9b, 9c, 10). These variations in accessory gene content and arrangement highlight the genetic diversity and evolutionary adaptation strategies among different β -CoVs. Adapted from (Chazal, 2021)

Table 2.2: Member Species of β -CoVs Classified by the ICTV According to the Most Recent Taxonomy. The Table Lists the Genus, Subgenus, Virus Name, Representative Isolate, GenBank Accession Number, Available Sequence, and Abbreviation

Genus	Subgenus	Species	Virus Name	Isolate	Accession Number	Available Sequence	Abbrev.
β -CoV	<i>Embecovirus</i>	<i>Betacoronavirus</i> <i>gravedinis</i>	Human coronavirus OC43	ATCC VR-759	AY585228	Complete genome	HCoV_OC43
	<i>Embecovirus</i>	<i>Betacoronavirus</i> <i>hongkongense</i>	Human coronavirus HKU1	HKU1	AY597011	Complete genome	HCoV_HKU1
	<i>Embecovirus</i>	<i>Betacoronavirus</i> <i>muris</i>	Murine hepatitis virus	A59	AY700211	Complete genome	MHV
	<i>Embecovirus</i>	<i>Betacoronavirus</i> <i>myodae</i>	Myodes rufocanus vole coronavirus 2/JL2014	RtMruf- CoV- 2/JL2014	KY370046	Complete genome	MrufCoV_2JL14

Table 2.2 continued

	<i>Embecovirus</i>	<i>Betacoronavirus ratti</i>	Betacoronavirus HKU24	HKU24-R05005I	KM349742	Complete genome	ChRCoV_HKU24
	<i>Hibecovirus</i>	<i>Betacoronavirus hipposideri</i>	Bat Hp-betacoronavirus/Zhejiang2013	Zhejiang2013	KF636752	Complete genome	Bat_Hp-BetaCoV
	<i>Merbecovirus</i>	<i>Betacoronavirus cameli</i>	Middle East respiratory syndrome-related coronavirus	HCoV-EMC	JX869059	Complete genome	MERS-CoV
	<i>Merbecovirus</i>	<i>Betacoronavirus erinacei</i>	Hedgehog coronavirus 1	2012-174/GER/2012	KC545383	Complete genome	EriCoV

Table 2.2 continued

	<i>Merbecovirus</i>	<i>Betacoronavirus pipistrelli</i>	Pipistrellus bat coronavirus HKU5	LMH03f	EF065509	Complete genome	Pi-BatCoV_HKU5
	<i>Merbecovirus</i>	<i>Betacoronavirus tyloncteridis</i>	Tyloncteris bat coronavirus HKU4	B04f	EF065505	Complete genome	Ty-BatCoV-HKU4
	<i>Nobecovirus</i>	<i>Betacoronavirus cororeum</i>	Rousettus bat coronavirus	GCCDC1 356	KU762338	Complete genome	Ro-BatCoV_GCCDC1
	<i>Nobecovirus</i>	<i>Betacoronavirus eidoli</i>	Eidolon helvum bat coronavirus CMR704-P12	CMR704-P12	MG693168	Complete genome	Ei-BatCoV_C704
	<i>Nobecovirus</i>	<i>Betacoronavirus rousetti</i>	Rousettus bat coronavirus HKU9	BF_005I	EF065513	Complete genome	Ro-BatCoV_HKU9

Table 2.2 continued

	<i>Sarbecovirus</i>	<i>Betacoronavirus pandemicum</i>	Severe acute respiratory syndrome coronavirus	Tor2	AY274119	Complete genome	SARS-CoV
	<i>Sarbecovirus</i>	<i>Betacoronavirus pandemicum</i>	SARS coronavirus	PC4-227	AY613950	Complete genome	SARS-CoV
	<i>Sarbecovirus</i>	<i>Betacoronavirus pandemicum</i>	Severe acute respiratory syndrome coronavirus 2	Wuhan- Hu-1	MN908947	Complete genome	SARS-CoV-2
	<i>Sarbecovirus</i>	<i>Betacoronavirus pandemicum</i>	Severe acute respiratory syndrome- related coronavirus	BtKY72	KY352407	Complete genome	SARSr-CoV

2.3.2 Structural Organization of CoVs

Coronaviruses (CoVs) are enveloped, positive-sense single-stranded RNA viruses with a well-defined structural organization that facilitates their replication and interaction with host cells (Figure 2.6). Their structure consists of four main structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N), along with a lipid bilayer derived from the host cell membrane that encapsulates the viral genome (Figure 2.7).

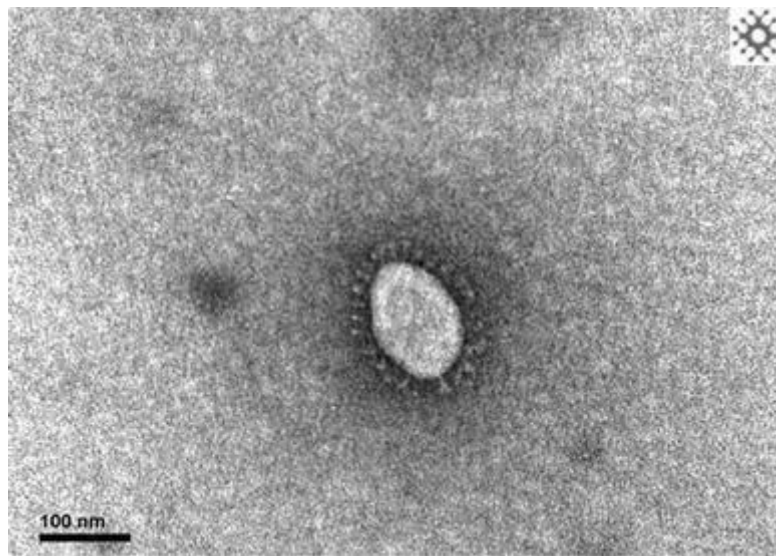


Figure 2.6: Electron micrograph of a CoV particle found in *Rhinolophus hipposideros* bat (Rihtarič et al., 2010)

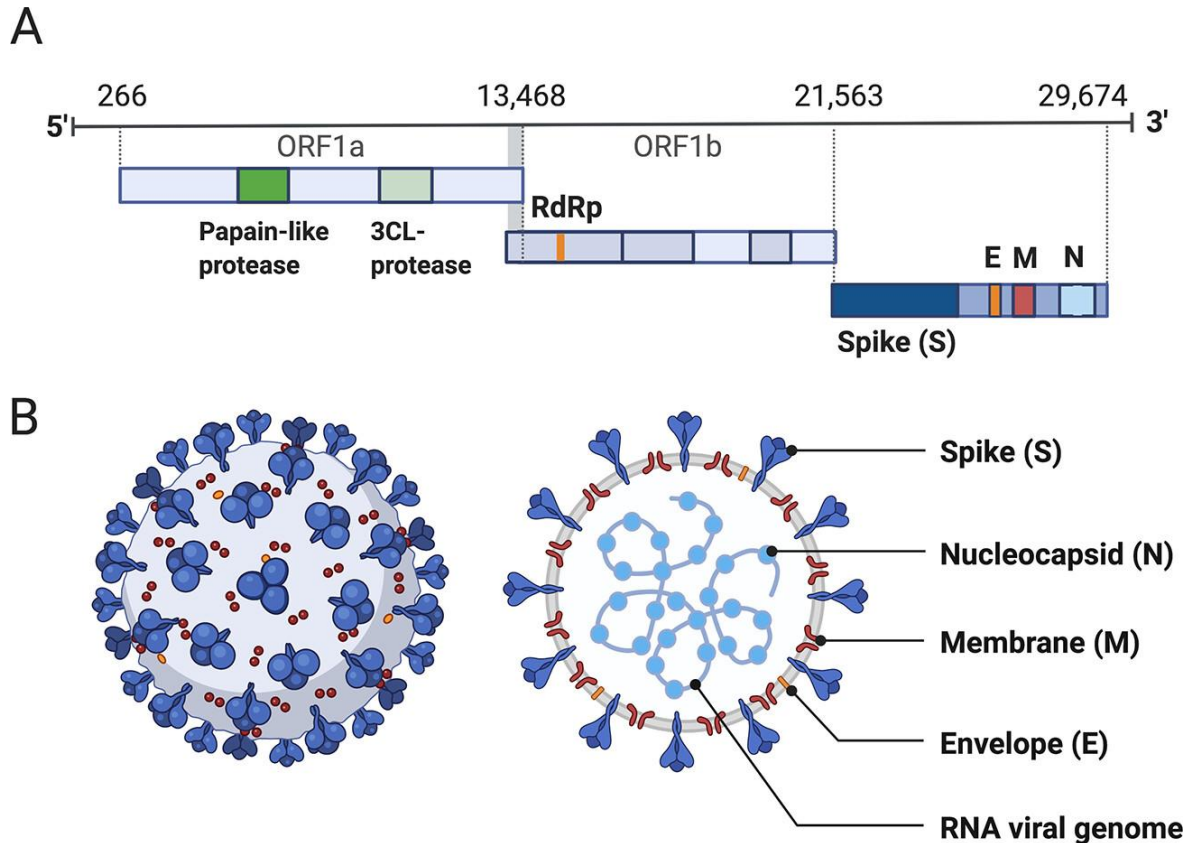


Figure 2.7: Structure of CoV genome (A) The CoV genome follows a highly conserved organization, consisting of three main regions. Open reading frames (ORFs) 1a and 1b encode two polyproteins that produce nonstructural proteins (nsp), including key enzymes such as RNA-dependent RNA polymerase (RdRp). The final third of the genome encodes structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. Additionally, accessory genes may be distributed throughout the genome. (B) The physical composition of the CoV virion, shaped by the conserved structural proteins S, E, M, and N (Rando et al., 2021)

The name “coronavirus” is derived from the Latin word *corona*, meaning “crown” or “halo”. This name was given due to the virus's distinct appearance under an electron microscope, where the S proteins protruding from the viral envelope resemble a solar corona (Almeida & Tyrrell, 1967). These spike projections are essential for viral entry into host cells and are a defining feature of CoVs. The first CoV were identified in the 1960s, with HCoV-229E and HCoV-OC43 being among the earliest discovered (Tyrrell et al., 1965). Over the

years, CoVs have been classified into four genera: α -CoV, β -CoV, γ -CoV, and δ -CoV, with many strains infecting humans, mammals, and birds (Fehr & Perlman, 2015).

The S protein is a trimeric glycoprotein that protrudes from the viral envelope, forming the characteristic crown-like appearance of CoVs. This protein plays a crucial role in viral entry by binding to host cell receptors, such as angiotensin-converting enzyme 2 (ACE2) in SARS-CoV-2 (Li et al., 2003). The S protein is also the primary target for neutralizing antibodies and vaccines, making it a key focus in the development of therapeutic strategies against CoV infections (Walls et al., 2020).

The M protein is the most abundant structural protein and is responsible for maintaining the shape and stability of the virus. It spans the lipid bilayer multiple times and interacts with other structural proteins, particularly the S, E, and N proteins, to facilitate viral assembly and budding (Neuman et al., 2006). The M protein plays an essential role in ensuring the proper formation of the viral envelope, contributing to the overall structural integrity of the virus.

The E protein is the smallest of the structural proteins, yet it plays a significant role in the virus's life cycle. It is involved in virus assembly, budding, and host cell interaction, and it also functions as an ion channel that can influence the pathogenicity of the virus (Schoeman & Fielding, 2019). Although present in relatively small amounts, the E protein contributes to the virus's ability to manipulate the host cell environment and facilitate efficient replication.

Inside the viral envelope, the N protein binds to the RNA genome, forming a ribonucleoprotein complex that protects the viral RNA and regulates its function. The N protein is highly conserved among CoVs and is involved in various stages of the viral

replication cycle, including genome packaging, transcription, and modulation of the host immune response (McBride et al., 2014). Due to its critical role in viral replication, the N protein is also a potential target for diagnostic assays and antiviral drugs.

At the core of the virus is its RNA genome, which is one of the largest among RNA viruses, typically ranging from 26 to 32 kilobases (kb) in length (Fehr & Perlman, 2015). This positive-sense RNA genome serves as messenger RNA (mRNA) for direct translation of viral proteins in host cells. It encodes not only the structural proteins but also a variety of non-structural proteins that are essential for viral replication and host immune evasion. The large genome size and high recombination rate contribute to the adaptability and evolutionary diversity of CoVs, enabling them to infect a wide range of host species (Gorbalenya et al., 2020).

2.4 Historical Perspectives on CoVs

The first HCoVs, HCoV-229E and HCoV-OC43 were first identified in the mid-1960s, recognised as a cause of common cold. Generally, α -CoVs and β -CoVs cause respiratory illness in humans and gastroenteritis in animals. The seven CoVs that can infect people are HCoV-NL63, HCoV-229E, HCoV-OC43 and HKU1 induce only mild upper respiratory diseases whereas SARS-CoV, MERS-CoV and SARS-CoV-2 are highly pathogenic viruses cause severe respiratory syndrome in human (Forni et al., 2017; Su et al., 2016b). Based on current sequence databases, all HCoVs have animal origins: SARS-CoV, MERS-CoV, HCoV-NL63 and HCoV-229E are considered to have originated in bats; HCoV-OC43 and HKU1 likely originated from rodents. Currently, 7 of 11 ICTV-assigned α -CoV species and 4 of 9 β -CoV species were identified only in bats. Thus, bats are likely the major natural reservoirs of α -CoVs and β -CoVs (Woo et al., 2012a).

2.4.1 Case Studies of Notable Zoonotic Viruses

In the past two decades, the members of the family Coronaviridae have crossed the species barrier thrice, causing three major epidemics of pandemic potential (Dhama et al., 2020). These spillover events highlight the significant threat posed by zoonotic viruses, which can emerge unexpectedly from wildlife reservoirs and rapidly adapt to human hosts. The ability of CoVs to recombine, mutate, and exploit different host receptors has made them particularly adept at crossing species barriers (Ye et al., 2020). The emergence of these zoonotic CoVs has been linked to various ecological and anthropogenic factors, including increased human-wildlife interaction, habitat destruction, and global trade of live animals (Tang et al., 2022). Bats have been recognized as natural reservoirs for many CoVs, while intermediate hosts such as civets and camels have played a role in viral transmission to humans. The repeated emergence of these viruses underscores the need for continuous

surveillance, early detection, and global cooperation in mitigating the risks of future outbreaks (Cui et al., 2019).

The three major zoonotic CoV outbreaks are SARS, MERS, and SARS-CoV-2 or more commonly known as COVID-19 (Goyal et al., 2022). They have each demonstrated the devastating potential of these viruses. Their ability to cause severe respiratory illness, high mortality rates, and global disruption emphasizes the critical importance of studying their origins, transmission dynamics, and evolutionary adaptations (Jelinek et al., 2021; Liu et al., 2020; Zhou et al., 2021).

2.4.1.1 SARS-CoV

CoVs first gained widespread attention during the SARS epidemic in 2002–2003. The SARS outbreak began in Guangdong, China, in November 2002 and spread to Hong Kong by February 2003 and rapidly spread to over 30 countries, with more than 50% of cases occurring in the Asia-Pacific region (Stadler et al., 2003). Epidemiological studies revealed that the initial human cases were linked to restaurant workers who handled wild and exotic animals. A physician who treated patients with "atypical pneumonia" introduced the virus to Hong Kong, where it quickly spread to 26 countries through international air travel. By the end of the outbreak, 8,096 confirmed cases and 774 deaths were recorded, leading to a case fatality rate of 9.6%. It was later determined, through PCR tests and serological evidence, that SARS-CoV likely passed to humans through an intermediate host, such as the Himalayan palm civet or raccoon dog (Guan et al., 2003). These animals, however, were not the virus's natural hosts but the transmission most probably occurred in the live wet markets or through trade-related activities.

Efforts to trace the origins of SARS-CoV led to the discovery of CoVs in bats. Serological and molecular evidence suggested that four species of horseshoe bats (genus *Rhinolophus*) were likely the natural reservoirs of the virus. However, it took another decade before a bat-associated virus with 95% sequence similarity to SARS-CoV was isolated (Ge et al., 2013; Guan et al., 2003). This virus, named bat SL-CoV-WIV1, binds to human, civet, and Chinese horseshoe bat ACE2 receptors, confirming *Rhinolophus* species as the natural hosts for SARS-related CoVs (Ge et al., 2013; Hu et al., 2017; Lau et al., 2005, 2010, 2015; Rihtarič et al., 2010; Yang et al., 2013). Research has since shown that several bat genera, including *Hipposideros* and *Asseliscus*, also harbor Sarbecoviruses. These include *Hipposideros amiger*, *Hipposideros larvatus*, and *Asseliscus stoliczkanus*, as well as less common hosts like *Chaerephon plicatus*, *Miniopterus schreibersii*, and *Nyctalus leisleri* in Southeast Asia, China, and parts of Europe (Drexler et al., 2010; Hu et al., 2017; Lin et al., 2017; Wacharapluesadee et al., 2015). Further studies of bats in caves throughout China's Yunnan province have revealed a wide variety of SARS-related viruses. Several of these CoVs can attach to the human ACE2 receptor. Although there's some evidence that these viruses can infect humans, widespread outbreaks are unlikely due to limited human-to-human transmission (Hu et al., 2017).

2.4.1.2 MERS-CoV

MERS-CoV, a type of β -CoV, was first identified in Saudi Arabia in 2012 in patients experiencing acute pneumonia (de Groot et al., 2013; Zaki et al., 2012). Patients were mostly in contact with camels or have drunk unpasteurized camel milk. Evidence strongly suggests that dromedary camels transmitted the virus to these individuals, as both viral genomes and MERS-CoV antibodies were found in camels linked to the human cases (Hemida et al., 2014; Reusken et al., 2013). Several viral lineages exist in dromedary camels, including one nearly

identical to viruses found in human clinical samples (Haagmans et al., 2014; Sabir et al., 2016). Research indicates that MERS-CoV originated in bats, specifically *Pipistrellus*, *Neoromicia*, and *Nycteris* species (Annan et al., 2013; Corman et al., 2014; Cui et al., 2019; Ithete et al., 2013). While MERS-CoV sequences show five distinct clades, only a few bat-related viruses can utilize the human dipeptidylpeptidase 4 (DPP4) receptor necessary for viral entry (Cotten et al., 2014). Therefore, the specific bat virus that led to MERS-CoV remains unidentified (Cui et al., 2013; Lu et al., 2015).

Analysis suggests that genetic changes occurred in either bats or camels before transmission to humans, with the initial bat-to-camel transmission possibly occurring in sub-Saharan Africa (Corman et al., 2014). MERS-CoV in Arabian Peninsula camels may have originated from imported camels from the Greater Horn of Africa (Corman et al., 2014; Müller et al., 2014). Humans typically contract MERS-CoV through close contact with infected camels' nasal secretions (Azhar et al., 2014). While most human transmissions occur in the Arabian Peninsula, international travel has occasionally spread the virus, leading to outbreaks like the one in South Korea in 2015.

In humans, MERS-CoV often causes severe pneumonia with a 35% fatality rate. In contrast, camels experience mild upper respiratory tract infections (Adney et al., 2014). This difference might be due to the location of the DPP4 receptor (Raj et al., 2013). In camels, it's found in the nasal epithelium, while in humans, it's primarily in the lower respiratory tract, thus restricting viral replication to the lungs and limiting human-to-human transmission (Widagdo et al., 2019). Despite limited transmission between humans, sporadic outbreaks highlight the continued threat posed by MERS-CoV.

2.4.1.3 SARS-CoV-2 (COVID-19)

In late 2019, Wuhan, China experienced an outbreak of pneumonia cases with an unknown cause. Due to prior experience with SARS, scientists were able to quickly identify the virus responsible for the new pneumonia cases as a novel β -CoV, subsequently named SARS-CoV-2 by the ICTV. While early COVID-19 cases clustered around Wuhan's Huanan Seafood Market (Maxmen, 2022), making it an initial suspect, the market's role as the origin point is not definitive. The fact that no animal samples from the market tested positive for SARS-CoV-2 raises questions (Li et al., 2021). Furthermore, evidence suggesting the virus's presence in other regions prior to the Wuhan outbreak adds to the mystery surrounding its true origin (Beldomenico, 2023; Lytras et al., 2021; Poon & Peiris, 2020). This makes pinpointing the virus's origin a complex issue. The Huanan market may have been a site of amplification rather than the actual source of the zoonotic event (Andersen et al., 2020; Holmes et al., 2021).

The genomic sequence of the initial human SARS-CoV-2 virus shares 96.2% nucleotide identity with RaTG13, a BtCoVs found in *Rhinolophus affinis* bats in Yunnan Province (Li et al., 2021; Zhou et al., 2020). Similar viruses to SARS-CoV-2 also have been identified in pangolins seized from illegal wildlife traders (Lytras et al., 2021; Nga et al., 2022)). While pangolins were initially considered a potential intermediary host, the genetic similarity at the nucleotide level was not as strong as that observed between SARS-CoV-2 and some BtCoVs (only 85-92% compared to 96.2% with RaTG13) (Poon & Peiris, 2020). However, the significant amino acid identity (97.4%) in the spike protein, particularly within the receptor-binding motif, between pangolin COVs and SARS-CoV-2, warrants attention (Li et al., 2021). This similarity could be the result of convergent evolution, recombination events or other yet-unknown mechanisms (Wacharapluesadee et al., 2021; Zhou et al., 2021).

The possibility of pangolins playing a role, even if not as the direct intermediary, in the emergence of SARS-CoV-2 cannot be entirely ruled out (Huang et al., 2023)

RmYN02, another bat virus, shares 93.3% of its nucleotide sequence with SARS-CoV-2. In certain genomic areas, this similarity increases to 96%, exceeding even the similarity observed with RaTG13 (Li et al., 2021). RmYN02's discovery might shed light on a distinctive SARS-CoV-2 feature: four-amino-acid residues, Proline-Arginine-Arginine-Alanine, (PRRA) insertion within the spike protein, situated between the S1 and S2 subunits. This insertion forms a polybasic cleavage site recognizable by the furin protease. It's notable that RmYN02 also possesses a three-amino-acid (PAA) insertion at this location, although it does not create a furin cleavage site (Zhou et al., 2020). These insertions suggest the viral genome's flexibility, particularly in the receptor-binding protein. The viruses mentioned, including SARS-CoV, belong to the *Sarbecovirus* clade, known for frequent recombination (Andersen et al., 2020). This recombination might explain the high homology between the pangolin virus spike protein and that of SARS-CoV-2. While there is no evidence that SARS-CoV-2 is itself a recombinant, divergence dates estimate its lineage circulated in bats for decades (Boni et al., 2020). However, these estimates might be skewed by recombination events.

2.4.1.4 Novel CoV Transmitted from Bats to Swine

Bat-borne β -CoVs present a significant public health risk to humans. It is important to note that α -CoVs may also be concerning and should not be overlooked in favour of focusing solely on β -CoVs. Furthermore, transmission affecting livestock can result in substantial economic repercussions. A notable example is the Porcine Epidemic Diarrhea Virus (PEDV), initially identified in Europe in the 1970s, which re-emerged in 2010-2011,

causing widespread morbidity and mortality in pig farms globally (He et al., 2022). While the specific receptor for PEDV remains debated, it has demonstrated the ability to infect cells from pigs, humans, monkeys, and bats, indicating a broad host range (Liu et al., 2015). PEDV exhibits a closer genetic relationship to the bat-associated virus BtCoV/512/2005 than to TGEV, suggesting the possibility of PEDV originating from direct transmission from bats to other species (Huang et al., 2013).

In 2017, a novel virus emerged in pig farms in Guangdong Province, China, where the SARS epidemic originated (Bolles et al., 2011; Pan et al., 2017; Zhou et al., 2018a). This outbreak resulted in the death of 24,693 piglets. The causative agent was identified as a novel HKU2-related bat α -CoV, named swine acute diarrhoea syndrome CoV (SADS-CoV). Genetic analysis revealed that the viral sequence shares 98.48% identity with SADS-related CoVs found in *Rhinolophus* bats, providing strong evidence that the virus originated in bats (Zhou et al., 2018a). Although the specific receptor for SADS-CoV remains unidentified, its broad species tropism *in vitro*, encompassing various rodent and human cell lines, suggests that α -CoVs have the potential for interspecies transmission to humans (Edwards et al., 2020; Yang et al., 2019). Despite initial control, the virus reemerged in pig farms in southern China in February 2019 (Zhou et al., 2019). The frequent introduction of bat-associated viruses into pigs, combined with the tendency of CoVs for recombination, raises concerns that pigs may serve as mixing vessels for the creation of novel human pathogens, like the dynamics observed with influenza viruses (Wang et al., 2018).

2.5 The One Health Concept and Its Relevance to BtCoV Surveillance

The One Health concept is an integrative framework that recognizes the interconnectedness of human, animal, and environmental health (Destoumieux-Garzón et al., 2018). This approach has become particularly important in the context of EIDs, many of which are zoonotic in origin. Bats, as reservoirs of a wide range of viruses including CoVs, play a critical role within this framework. Their ecological importance as pollinators, seed dispersers, and insect controllers must be considered alongside their potential to harbor pathogens of relevance to human health (Calisher et al., 2006; Olival et al., 2017). Importantly, conservation of bat populations is not only essential for maintaining ecosystem services but also for reducing disruption-driven stress that may alter viral shedding dynamics (Brook & Dobson, 2015).

Regions with high biodiversity and frequent human–wildlife interaction, such as Sarawak, provide key contexts where the One Health approach is highly relevant. Cave tourism, agricultural expansion, and urban encroachment on bat habitats increase opportunities for viral circulation at the human–bat interface (Latinne et al., 2020). These pressures are particularly concerning in Southeast Asia, where rapid land-use change and dense human populations overlap with wildlife habitats, creating environments where pathogens can more readily circulate (Gottdenker et al., 2014; Morse et al., 2012). BtCoV surveillance in such areas contributes not only to understanding viral diversity but also to anticipating ecological and behavioral drivers that may shape opportunities for cross-species transmission.

By situating BtCoV research within a One Health perspective, surveillance efforts can be aligned with both conservation goals and public health preparedness. This holistic

approach emphasizes that effective monitoring and risk reduction require cross-disciplinary collaboration among ecologists, veterinarians, medical researchers, and policymakers (Gebreyes et al., 2014; Mackenzie & Jeggo, 2019). Furthermore, One Health frameworks promote the integration of ecological surveillance data with epidemiological and socioeconomic information, which can improve early-warning systems for disease emergence (Whitmee et al., 2015). In the context of Sarawak, embedding CoV surveillance within broader One Health initiatives can therefore strengthen both biodiversity conservation and pandemic preparedness in a region of global ecological significance.

2.6 Current Knowledge of Research on BtCoVs in Malaysia

All the epidemic strains of CoVs such as SARS-CoV, MERS-CoV, and SARS-CoV-2 are β -CoVs with SARS-CoV and SARS-CoV-2 from subgenus *Sarbecovirus* while MERS-CoV from subgenus *Merbecovirus*. All these CoVs have bat ancestry and are genetically related to CoVs isolated from bats (Han et al., 2019). The isolation of β -CoVs from Chiropterans, specifically insectivorous bats, is not novel and has been validated and revalidated by virologists worldwide (Lacroix et al., 2017; Lee et al., 2018; Lin et al., 2017; Monchatre-Leroy et al., 2017; Shehata et al., 2016; Tao & Tong, 2019; Wacharapluesadee et al., 2013).

Southeast Asia is home to approximately 378 bat species (Kuo et al., 2017; Tu et al., 2017), about 32% of which occur in Malaysia. In Peninsular Malaysia, 143 species have been recorded (Senawi & Ahmad, 2021). In Sarawak, at least 98 species have been documented, with recent surveys suggesting the number may approach or exceed 108 species across its diverse habitats (Kumaran et al., 2011). While they do inhabit and roost in cave systems such as Niah Cave, Mulu Cave, Wind Cave, and Fairy Cave, which are also popular

tourist attractions, many bat species also occupy forests, forest edges, and other natural habitats (Jinggong et al., 2022; Morni et al., 2018; Rosli et al., 2018). Additionally, many bat species are found roosting in human-interface areas, including urban parks, roof spaces, abandoned buildings, and fruit orchards near villages (Jinggong et al., 2022). These proximity-based roosting behaviors increase the likelihood of bat-human interactions, especially in regions where bats are in close contact with livestock, domestic animals, and human settlements (Ayivor et al., 2017; Suominen et al., 2023). As CoVs not only cause respiratory tract infections, but it is also an agent for gastroenteritis and being shed via feces (Termansen & Frische, 2023). The identification of β -CoV from bat guano in Khao Chong Phran non-hunting area in Ratchaburi province, Thailand had suggested the increased biorisk of transmission from the bat guano and urine contaminated environmental to human such as the guano miners and tourists (Wacharapluesadee et al., 2013).

There is no doubt now bats are natural hosts for CoVs and serve as potential source of spillover from animals to humans (García-Sastre & Richt, 2019; Wacharapluesadee et al., 2018; Wang & Anderson, 2019; Yang et al., 2016). However, other animals such as boars (Wang et al., 2018), civets (Wacharapluesadee et al., 2018), snake (Ji et al., 2020), pangolins (Liu et al., 2019) etc. are competent hosts for CoVs. These animals are commonly hunted and butchered for bushmeat in Sarawak and are readily available at the fresh markets in the rural towns and villages (Cantlay et al., 2017). Although illegal to a certain extent, bushmeat is an important source of protein and income for the indigenous communities (Kurpiers et al., 2015; Lee et al., 2020). Bushmeat consumption is also believed to have some unproven medicinal properties that plays a role in the sustainability of this business (Wolfe et al., 2005). For instance, pangolin meat is believed to relieve rheumatism and improve blood circulation while its bile improves vision (Li et al., 2020).

There are currently no reported cases of acute respiratory syndrome (ARS) linked to the hunting and butchering of wildlife in Sarawak but if any would be underreported due to the absence of viral diagnostic capability of the laboratories in Sarawak and probably the rest of Malaysia (Toh et al., 2025). The only prevalence study of ARS was published by a Singaporean group in collaboration with local hospitals in Sarawak and they have defined the prevalence of influenza virus, adenoviruses, human enteroviruses, HCoVs, respiratory syncytial virus and parainfluenza virus (Toh et al., 2019). The first syndromic study in Sarawak by foreign researchers is not surprising, given the lack of national research funding to support prevalence studies (Toh et al., 2019, 2025). Despite this limitation, research efforts have continued to shed light on bat-borne viruses in Malaysia. A study on bat viruses in Gunung Mulu National Park, Sarawak, led to the discovery of *Sarawak mobatvirus*, a novel hantavirus within the genus *Mobatvirus* (Zana et al., 2019). Additionally, recent studies in Malaysia have revealed a diverse range of CoVs circulating in bat populations (Table 2.3). In Sarawak, both α -CoVs and β -CoVs were detected in *Penthetor lucasi* (Dusky fruit bat) and insectivorous bats, with some strains forming novel clades (Habeebur-Rahman et al., 2023; Tan et al., 2021). Beyond Sarawak, other studies have also identified bat-associated CoVs in different regions of Malaysia. In Sabah, both α -CoVs and β -CoVs were discovered in cave-roosting bats, i.e. *Hipposideros cervinus* and *Rhinolophus creaghi*, suggesting that bat populations in limestone cave ecosystems may serve as reservoirs for a wide range of CoVs (Mahyudin et al., 2021). Similarly, in Krau Wildlife Reserve, Pahang, a study detected SARS-CoV-2-like viruses in insectivorous bats, revealing their potential role in the evolution of zoonotic CoVs (Siew et al., 2023). In east coast Malaysia, SARS-CoV-2-like viruses were identified in 3% of sampled bats, exhibiting a 98-99% sequence identity to human SARS-CoV-2 (Gilbert et al., 2023).

Table 2.3 Compilation Of Studies On BtCoVs In Malaysia. The Table Details The Type Of Research Conducted, Sample Types Collected, Localities Surveyed, Detection Methods Applied, Major Findings, Year Of Publication, And Corresponding References

Research	Sample Type	Location	Method	Finding	Year	Reference
Ecological Surveillance of Bat Coronaviruses in Sarawak	Bat guano	Sarawak	Hemi-nested PCR using primers targeting partial RdRp	Detection of six α -CoVs and four β -CoVs in guano, with a prevalence of 47.6% (n = 10/21)	2021	(Tan et al., 2021)
Detection of Viral and Bacterial Pathogens in Bats from Sabah, Malaysia	Insect and fruit bats (rectal and throat samples)	Sabah	RT-PCR using consensus primers	Detection of three α -CoVs and two β -CoVs from <i>R. creaghi</i> and <i>H. cervinus</i>	2021	(Mahyudin et al., 2021)
Detection of Novel Coronaviruses from Dusky Fruit Bat (<i>Penthetor lucasi</i>) in Sarawak, Malaysian Borneo	Dusky fruit bats (<i>Penthetor lucasi</i>) guano	Sarawak	Hemi-nested PCR using primers targeting partial RdRp gene using Watanabe et al., 2010	Detection of two genera, α -CoV and β -CoV, with the bat CoV positivity rate was 38.5% (n = 15/39)	2023	(Habeebur-Rahman et al., 2023)

Table 2.3 continued

Presence of SARS-CoV-2-Like Coronaviruses in East Coast Malaysia	Insect and fruit bats (oropharyngeal samples)	East Coast Malaysia (Kelantan, Terengganu and Pahang)	Nested and hemi-nested using primers targeting partial 3'UTR gene using Holbrook et al., 2021; Quan et al., 2010 and designed primers	Detection of SARS-CoV-2-like viruses in 3% of bats. Sequences showed 98-99% similarity to human SARS-CoV-2	2023	(Gilbert et al., 2023)
Bat Coronavirus was Detected Positive from Insectivorous Bats in Krau Wildlife Reserve Forest	Insect and fruit bats (oral and fecal samples)	Pahang	RT-PCR using primers from (Poon & Peiris, 2008)	Detection of bat CoV in <i>Rhinolophus</i> spp. that may represent a distinct lineage	2023	(Siew et al., 2023)

These findings highlight the ongoing need for comprehensive surveillance and funding to better understand the zoonotic potential of bat-associated viruses in Malaysia, particularly in regions with high bat diversity such as Sarawak. The detection of novel CoVs and their close genetic links to known epidemic strains further emphasize the necessity of continued research, particularly in cave ecosystems and other bat roosting sites, which may harbor undiscovered viral lineages.

2.7 Molecular Detection Methods of BtCoVs

The surveillance of BtCoVs is critical for understanding their diversity, evolution, and potential for zoonotic spillovers. BtCoV surveillance has been geographically uneven, with a concentration of studies in China and notable gaps in South Asia, the Americas, sub-Saharan Africa, and East Africa before the COVID-19 pandemic (Cohen et al., 2023). This disparity highlights the need for expanded surveillance efforts in underrepresented regions to gain a more comprehensive understanding of the global distribution of BtCoVs. Since the pandemic, there has been progress in addressing these gaps, with recent surveillance efforts reported in Latin America and Madagascar (Cohen et al., 2023).

Surveillance of bat-borne CoVs in Southeast Asia faces several important limitations that constrain the reliability and comparability of available data. First, significant geographic gaps remain, with most studies concentrated in a few accessible locations, while many ecologically important areas such as remote cave systems and forest interiors are undersampled (Anthony et al., 2017; Latinne et al., 2020). This uneven coverage limits the ability to generate representative prevalence estimates across the region. Second, many laboratories face limited diagnostic capacity, particularly in low- and middle-income countries (LMICs), where access to high-throughput sequencing platforms and advanced

molecular tools is restricted (Wacharapluesadee et al., 2015). This often results in reliance on a narrow set of conventional PCR assays that may miss novel or divergent viral lineages. Third, underreporting remains a concern, as negative or inconclusive results are less frequently published, creating a bias toward studies that only highlight positive detections (Mulemena et al., 2025). This skews the regional picture of viral circulation. Fourth, there is a lack of standardized protocols for sampling, RNA extraction, and PCR assays, which complicates cross-study comparisons and may partly explain the wide variation in reported prevalence values (Lacroix et al., 2017; Olival et al., 2017).

Molecular detection methods remain the cornerstone of BtCoV surveillance, enabling researchers to identify, classify, and monitor the genetic diversity of these viruses with high sensitivity and specificity. Among these, reverse transcription polymerase chain reaction (RT-PCR), next-generation sequencing (NGS), and hybrid capture approaches are the most used techniques, each offering unique advantages in virus detection and characterization. RT-PCR is widely applied due to its sensitivity in amplifying conserved CoV genomic regions, while NGS allows for unbiased, high-throughput sequencing of entire viral genomes, facilitating the discovery of novel CoVs. Meanwhile, the hybrid capture approach enhances viral genome recovery by selectively enriching target sequences, improving detection sensitivity even in samples with low viral loads (Briese et al., 2015)

A recent study reviewed 127 articles evaluate molecular studies on CoVs in bats (Mulemena et al., 2025).. Notably, 85 out of 127 articles (67%) were published between 2016 and 2022, reflecting a growing interest in this field during recent years. These studies originated from 54 different countries, with China contributing 26% (33 out of 127) of the total publications. Among the studies analyzed, 94.5% (120/127) utilized PCR assays

targeting the partial RdRp gene, generating sequences ranging from 123 to 440 bp. Other genetic targets included helicase (0.8%, 1/127), spike (S) gene (7%, 9/127), nucleocapsid (N) gene (2.4%, 3/127), envelope (E) gene (2.4%, 3/127), and membrane (M) gene (0.8%, 1/127) (Mulemena et al., 2025)..

The same study further reported that full-genome sequencing was conducted in 33.9% (43/127) of the studies, while a metagenomic approach was employed in 15.7% (20/127) (Mulemena et al., 2025). Notably, several studies that performed full genome sequencing initially relied on RdRp gene amplification for preliminary detection. Regarding primer usage, 55.9% (71/127) of studies applied specific target-sequence primers, also referred to as consensus primers. In contrast, 5.5% (7/127) employed sequence-independent single primer amplification. The remaining 38.6% (49/127) did not specify the type of primers used (Mulemena et al., 2025.).

Despite these advancements, effective surveillance is hindered by logistical and technical challenges, particularly in field-based sample collection and processing. The success of molecular detection depends on factors such as sample type, preservation methods, and laboratory conditions, all of which can impact viral RNA integrity and detection efficiency (Anthony et al., 2017b). Standardized protocols for sample storage, RNA extraction, and sequencing library preparation are essential for generating reliable, comparable data across different surveillance programs. Furthermore, the integration of serological assays with molecular techniques can provide complementary insights, helping to identify past exposures and viral circulation dynamics within bat populations.

2.7.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Global efforts to detect BtCoVs have involved a range of molecular techniques, with a strong emphasis on RT-PCR (Tan et al., 2023). The process begins with reverse transcription, converting RNA into complementary DNA (cDNA), which is then amplified through PCR using primers that target conserved CoV sequences. Regions such as RdRp and the spike protein have proven effective targets for identifying BtCoVs in fecal and intestinal samples (Shirato et al., 2011; Su et al., 2016). Universal RT-PCR assays designed to amplify highly conserved regions have facilitated the discovery of novel BtCoVs, reinforcing the role of bats as key viral reservoirs (Poon & Peiris, 2008). Following amplification, the resulting DNA can be detected through gel electrophoresis or real-time PCR, the latter providing quantitative viral load measurements (MacKenzie & Smith, 2020). The success of RT-PCR depends on primers, a short DNA sequences that bind to specific regions of the viral genome to initiate amplification.

Pathogens have been identified and tracked globally through diverse detection and surveillance techniques, including molecular approaches such as genomic surveillance. These molecular methods typically focus on analysing key genetic sequences in pathogens, particularly those encoding structural proteins like the S glycoprotein, E, M, and N proteins, as well as non-structural genes involved in viral replication, such RdRp, helicase, and open reading frames ORF1a and ORF1b (Chu et al., 2008). Approximately 55.9% of studies employed target sequence-dependent primers, designed for species-specific, genus-specific, or broad-range detection (Alves et al., 2022; Chu et al., 2006; Guo et al., 2021; Han et al., 2019). While these assays aim for conserved regions, the significant genetic diversity among CoVs might limit the sensitivity of existing primers, potentially reducing their capacity to detect a wider spectrum of viruses (Anantharajah et al., 2021; Dong et al., 2021).

In fact, given the vast diversity of CoVs, including those yet to be discovered, it is difficult to design primers that can amplify and capture the full diversity of CoVs (Tan et al., 2023). However, the immense diversity of CoV poses a significant challenge for primer design, as universal degenerate primers may underestimate the true prevalence and diversity of circulating viruses. Moreover, directly retrieving viral genomes from animal samples remains a significant laboratory challenge. The presence of host tissues and microbiota introduces a high amount of background genetic material, which dilutes viral genome fragments and necessitates a much greater sequencing depth for effective detection and accurate genotyping (Fitzpatrick et al., 2021; Houldcroft et al., 2017). While RT-PCR is highly sensitive, it requires careful execution to avoid false-positive results (Verna et al., 2021). Additionally, it demands specialized equipment and trained personnel, making its application resource-intensive but indispensable for viral surveillance and epidemiological studies (Verna et al., 2021).

2.7.2 Next-Generation Sequencing (NGS)

NGS technologies have emerged as powerful tools for characterizing BtCoVs, with whole-genome sequencing (WGS) enabling the discovery of novel viruses and providing insights into their evolutionary dynamics (Bolles et al., 2011). Viral genomes exhibit diversity due to potentially high mutation rates associated with RNA replication. Furthermore, the frequent occurrence of recombination events contributes to the emergence of new viral variants. The high error rate of the viral RdRp gene and the frequent occurrence of recombination events contribute to the genetic diversity of CoVs (Bolles et al., 2011).

2.7.3 Hybrid Capture Approach

The development of targeted genomic sequencing methods, such as hybrid-capture approaches, represents a significant advancement in BtCoV surveillance (Kuchinski et al., 2022). Hybrid-capture methods offer advantages over amplicon-based sequencing, providing a more comprehensive and unbiased assessment of viral diversity (Kuchinski et al., 2022). These methods involve the use of oligonucleotide probes to selectively capture viral sequences from complex samples, followed by high-throughput sequencing. This approach is particularly useful for identifying divergent CoVs that may not be efficiently amplified by PCR-based methods.

2.8 Serological Detection Methods

Serological assays detect antibodies against CoV in bat serum (Arteaga et al., 2023). These assays indicate prior exposure and can elucidate the prevalence of CoV infections in bat populations. Common assays include ELISA (Enzyme-Linked Immunosorbent Assay) and virus neutralization test (VNT).

2.8.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs detect antibodies that bind to viral antigens. Bat serum samples are incubated with viral antigens, and then antibodies are detected using enzyme-linked secondary antibodies (Arteaga et al., 2023). The amount of antibody present is proportional to the signal generated by the enzyme (Bond et al., 2020). Serological assays are useful for retrospective studies and for understanding the long-term dynamics of CoV infections in bats (Zhang et al., 2021). They can also help identify which viruses bats have been exposed to, which can inform risk assessments (Bond et al., 2020). Serological assays can be less sensitive than RT-PCR. They may not be able to detect recent infections, as it takes time for antibodies to

develop (Zhang et al., 2021). Cross-reactivity between different CoVs can also lead to false-positive results (Zheng et al., 2022).

2.8.2 Virus Neutralization Test (VNT)

VNTs measure the ability of antibodies to neutralize the virus and prevent it from infecting cells. Bat serum samples are incubated with live virus, and then the mixture is added to susceptible cells. If the antibodies in the serum neutralize the virus, the cells will not become infected (Wong et al., 2019).

2.9 PCR Assays Utilized in This Study: Quan-Assay vs Watanabe-Assay

Early and accurate detection of BtCoVs is critical for identifying potential threats of zoonotic spillover. RT-PCR assays are highly sensitive and specific, making them the preferred method for detecting viral RNA. Typically, molecular detection of CoVs uses degenerate primers that target conserved regions of the RdRp gene, which helps to minimize false negatives (Drzewnioková et al., 2021; Holbrook et al., 2021; Hu et al., 2018; Xiu et al., 2020). This gene is widely regarded as an optimal target for CoV detection, including SARS-CoV-2-like viruses, as this gene is highly conserve across CoVs while still containing sufficient sequence variation to differentiate between species and strains, ensuring both broad detection and good specificity (Abbasi et al., 2021). RT-PCR assays targeting RdRp gene also demonstrate high analytical sensitivity, capable of detecting small quantities of viral RNA, which is why the WHO recommended RdRp-based assays as first-line or confirmatory tests during the early stages of the COVID-19 pandemic, given their superior sensitivity compared to other targets such as the E or N genes (Quino et al., 2021). Furthermore, this gene encodes a critical enzyme essential for viral genome replication, making it a functionally important and stable region of the viral genome (Uengwetwanit et

al., 2022). Incorporating RdRp into multiplex assays alongside other targets enhances diagnostic accuracy by reducing false positives and negatives that may arise from primer-probe cross-reactivity or viral mutations (Tombuloglu et al., 2022). Collectively, these features make the RdRp gene a robust and reliable molecular marker for CoV surveillance and diagnostics.

The Quan and Watanabe protocols are frequently used pan-CoV PCR assays for detecting BtCoVs (Quan et al., 2010; Watanabe et al., 2010). While the Quan-assay (Q-assay) offers broad detection across multiple bat species, the Watanabe-assay (W-assay) is designed to target specific viral lineages. In 2010, Quan et al. identified a novel β -CoV in a Commerson's leaf-nosed bat (*Hipposideros commersoni*) in Nigeria. Using molecular techniques, they characterized the virus and compared it to known CoVs, revealing that African bats could serve as reservoirs for SARS-like CoVs, much like their counterparts in Asia (Quan et al., 2010). At the time, research on SARS-related coronaviruses (SARS-rCoVs) was predominantly focused on Asia, making this discovery particularly significant. It emphasized the need to expand surveillance beyond Asia to fully understand the global diversity and distribution of these viruses.

Similarly, in 2008, researchers in the Philippines screened 52 bats samples for CoVs RT-PCR, detecting viral RNA in 55.8% of the samples (Watanabe et al., 2010). They identified sequences from two major CoV groups: Group 1 (genus α -CoV) and Group 2 (genus β -CoV), both of which showed genetic similarities to CoVs previously detected in bats from China. These findings underscored the endemic presence of BtCoVs in the Philippines, further highlighting the widespread nature of these viruses across different regions (Tsuda et al., 2012). Subsequent research expanded on this work by implementing

similar protocols across various regions, including Southeast Asia. While some studies utilized the Q-assay and W-assay independently, others integrated both assays for a comprehensive analysis (Anthony et al., 2017b; Cappelle et al., 2021; Febriani et al., 2018; Habeebur-Rahman et al., 2023; Holbrook et al., 2021; Huong et al., 2020; Kandeil et al., 2023; Kumakamba et al., 2021; Lange et al., 2023; Latinne et al., 2020; Lee et al., 2020; Liu et al., 2024; Nga et al., 2022; Onyuok et al., 2019; Tan et al., 2021; Valitutto et al., 2020; Wacharapluesadee et al., 2015, 2020, 2021, 2024; Xu et al., 2019). These combined approaches likely aimed to increase the sensitivity and breadth of detection, capturing a wider range of CoVs (Xu et al., 2019).

The Q-assay and W-assay were selected due to their established use in pan-CoV surveillance across diverse ecological settings. Despite their widespread use in various ecological settings, these assays have not been thoroughly explored in Sarawak. The combined use in this study allows for cross-validation of detection results, broader coverage of CoV diversity, evaluation of assay performance in a previously untested region (Sarawak). This is relevant, as Southeast Asia, including Sarawak, is considered a high-risk area for emerging infectious diseases due to its large, intact natural habitats and ongoing ecosystem fragmentation, which often results from logging, agricultural expansion, and land-use change. Such fragmentation not only destroys or degrades habitats but also increases human–wildlife interactions, thereby elevating the risk of zoonotic spillover (Evans et al., 2023).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Size Calculation

The expected prevalence of BtCoVs can vary significantly based on species, regions, and sampling methods. Studies have found BtCoV prevalence rates ranging from 1% to 50%, depending on the bat population and geography (Meyer et al., 2024; Warmuth et al., 2023). In Southeast Asia, including Borneo, prevalence rates typically fall between 5% and 15% in many surveyed bat populations (Guillebaud et al., 2025; Mulemena et al., 2025; Tan et al., 2021). Certain bat species are known to have higher CoV carriage rates. For example, the horseshoe bats (*Rhinolophus spp.*) often show higher CoV prevalence, sometimes exceeding 20% (Latinne et al., 2020). Whereas fruit bats (*Pteropus spp.*) may show lower prevalence, often around 5%-10% (Guillebaud et al., 2025). Research in Malaysia, including Sarawak, has reported prevalence rates between 6% and 15%, depending on the detection method (e.g., PCR assays) and seasonal variations (Gilbert et al., 2023; Habeebur-Rahman et al., 2023; Tan et al., 2021).

The required sample size for this prevalence study was calculated using the standard formula (Daniel, 1999), with an expected prevalence ranging from 5% to 20%.

$$n = \frac{Z^2 p(1 - p)}{d^2} \quad \text{Equation 3.1}$$

Parameter:

p=0.05 & 0.20 (5% & 20% of prevalence)

Confidence level = 95% (Z=1.96)

Desired margin of error (d) = 0.05 (±5%)

Calculation:

If the prevalence is 5%,

$$n = \frac{(1.96^2)0.05(1 - 0.05)}{0.05^2} = 72.99 = 73 \quad \text{Equation 3.2}$$

If the prevalence is 20%,

$$n = \frac{(1.96^2)0.20(1 - 0.20)}{0.05^2} = 245.8 = 246 \quad \text{Equation 3.3}$$

Based on an expected prevalence range of 5% to 20%, the estimated minimum required sample size is 73, while the maximum is 246. In this study, a total of 346 samples were collected, exceeding the maximum estimated requirement.

3.2 Study Sites

A series of field trips were conducted across selected localities in Sarawak to collect bat samples, targeting sites that reflected both ecological diversity and human–animal interfaces. These included caves with visitor access, national parks with overlapping ecotourism and bat habitats, and orchards where fruit bats foraged near communities. Sampling was carried out in the western regions (Wind Cave Nature Reserve, Kampung Bobak, Kampung Peros, Kampung Gayu, and Kampung Merdang Gayam) and Northern regions (Niah National Park and Mulu National Park) (Appendix D).

Niah NP and Mulu NP were prioritized for their large cave systems hosting colonies of *Rhinolophus*, *Miniopterus*, and *Hipposideros* species, as well as their high tourist activity. In contrast, western sites represented more disturbed habitats such as orchards and forest

edges. This mix of pristine and human-modified environments enabled comparative insights into BtCoV presence across different ecological and anthropogenic contexts.

To provide a clear overview of the methodological framework, a flowchart summarizing the experimental design and workflow is presented (Figure 3.1). This schematic illustrates the sequential steps undertaken in the study, beginning with bat sampling and fecal collection, followed by laboratory analyses including RNA extraction, PCR screening, and sequencing, and concluding with phylogenetic analyses and data interpretation. The inclusion of this visual summary enables readers to follow the structure of the study design more intuitively before engaging with the detailed methodological descriptions that follow.

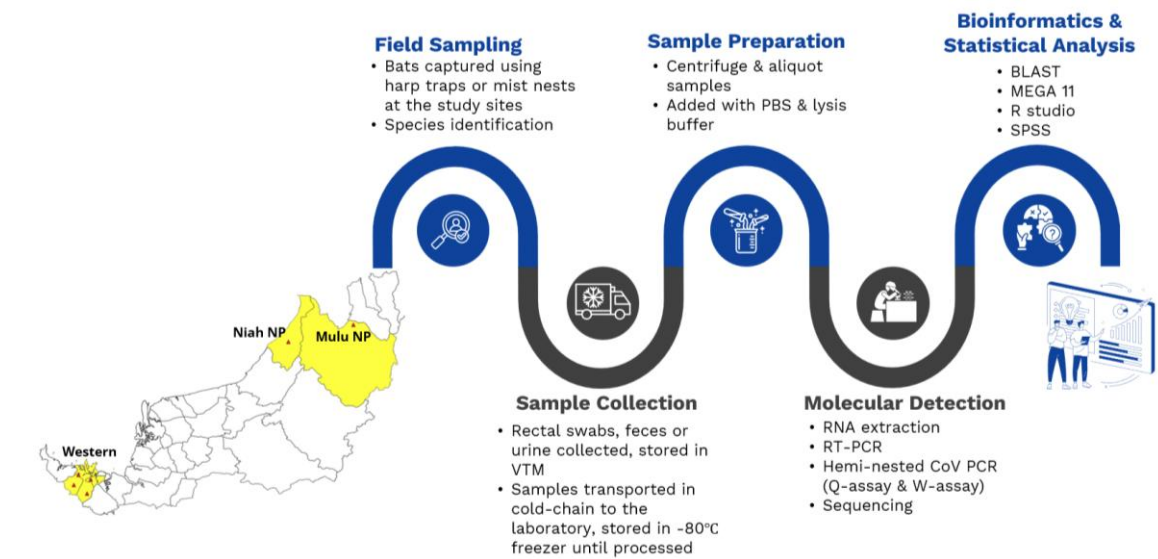


Figure 3.1: Study workflow and sampling sites in Sarawak, Malaysian Borneo. The flowchart outlines the main steps of the study: field sampling, sample collection and preparation, molecular detection, bioinformatics and statistical analysis. The map shows the three sampling regions: Western Sarawak, Niah NP, and Mulu NP

3.2.1 Bat Sampling and Sample Collection

Sampling was conducted between 2021 and 2023, covering the western regions (September 2021–January 2022) and the northern regions, Niah NP (November 2022) and Mulu NP (June 2023). Bats were captured from their natural environments, including orchards and protected areas such as national parks, in Sarawak's western and northern regions. Sampling was conducted from 1800 to 0600 hours using harp traps and mist nets. A total of four harp traps and 10 mist nets were deployed during sampling. Captures techniques varied based on the species being targeted and the location where the samples were collected. Mist nets were particularly effective for frugivorous bats, such as *Cynopterus* and *Penthetor*, which fly along orchard edges and forest trails. Harp traps were more suitable for insectivorous species, especially in flyways such as cave entrances, forest trails, and riverine corridors, where bats often commute at low heights. To minimize possible cross-contamination among bats captured in the same harp trap, several precautions were taken. Each bat was removed from the trap immediately after detection and placed into an individual, clean cotton holding bag with draw-string mouth and kept in a cool dry place. The cotton bags were never reused on the same night, and after each use they were washed, disinfected, and dried before the next sampling. The sampling team changed gloves between handling individual bats to avoid contamination of samples. In addition, all sampling equipment (e.g., calipers, scales) was disinfected with 70% ethanol between bats. These measures ensured that samples collected from different individuals were not compromised by contamination from other bats in the same harp trap.

Bats were weighed in grams in the bags using a Pesola Spring balance, and once the bat was removed from the bag for sampling, the bag was re-weighed and its weight subtracted from the previous total. Species identification and sex determination were carried

out directly in the field following Payne et al., (1985) and Phillipps & Phillipps, (2018). The bats were removed from the bag and the desired samples were collected, which are rectal swabs, feces, and urine. The order of sampling varied. For example, urine may have been expelled during initial handling, making it the first sample collected. Urine was collected opportunistically during handling. Each bat was gently held in a clean plastic bag to capture any urine released. The urine droplets were then transferred immediately into a sterile microcentrifuge tube using a sterile pipette tip. The bag was checked for fresh feces before continuing, and if fresh feces were available, they were used as a sample, the need for a rectal swab was not necessary. Bats were not held for longer than six hours. Frugivorous bats were given 100% fruit juice or sugar water prior to release. To minimize the likelihood of recapturing the same individuals, bats were temporarily marked by coloring their nails with non-toxic nail polish before release. This marking remained visible during the sampling period, allowing field teams to recognize and avoid resampling the same bat.

Specimens were temporarily stored in standard refrigerators or freezers available at the accommodation facilities used during fieldwork. The samples were transported in a cold chain to the laboratory and stored in a -80°C freezer until processed. All specimens were handled according to the recognized procedures for animal care and use recognised by Universiti Malaysia Sarawak Animal Ethics Committee (UNIMAS-AEC).

3.3 Permit Approval and Ethical Considerations

This study was conducted in compliance with all relevant ethical guidelines and legal requirements for wildlife research. Necessary permits for bat sampling, handling, and sample collection were obtained from the appropriate authorities, Sarawak Forestry Corporation (SFC) (Appendix B). Ethical approval for this research was granted by Universiti Malaysia

Sarawak Animal Ethics Committee (UNIMAS-AEC), following established protocols for the humane treatment of animals in research (Appendix C). All procedures, including bat handling, sample collection, and release, were designed to minimize stress and avoid harm to the animals. Standard operating procedures were followed to ensure that bats were handled gently and released promptly after sample collection.

To further minimize distress, bats were retained for a maximum of six hours prior to release. To maintain hydration and energy, frugivorous species were supplemented with either fruit juice or sugar solution to support hydration and energy balance. All capture and sampling methods adhered to internationally recognized ethical guidelines, such as those established by the American Society of Mammalogists (Sikes et al., 2016) and the guidelines for bat research published by the Chiropteran Research and Conservation Network. Additionally, biosafety measures were strictly implemented to prevent cross-contamination and ensure the safety of both researchers and wildlife. Personal protective equipment (PPE), including gloves and masks, was worn during handling, and all equipment was sterilized between uses. Proper disposal or decontamination of used sampling materials was carried out according to laboratory biosafety protocols. This research was conducted with the utmost regard for conservation principles, ensuring that the study did not negatively impact bat populations or their habitats.

3.4 Molecular Analysis

The RdRp gene was selected as the molecular target for CoV detection in this study because it is well conserved across CoV genera, while still retaining sufficient sequence variability to distinguish between species and strains. This conserved nature allows for broad-range detection of both known and novel CoVs, which is particularly advantageous in

wildlife surveillance where uncharacterized viral diversity is expected (Hu et al., 2018). In contrast, genes such as the S and N exhibit greater sequence variability, although informative for host specificity, tissue tropism, and pathogenicity, limits their utility in pan-CoV detection assays (Chan et al., 2020).

Both the Q-assay and W-assay targeted RdRp gene, which is conserved among CoVs, but differed in primer sets and cycling conditions. Comparing the performance of these assays allowed for a more comprehensive detection approach, increasing the likelihood of identifying diverse CoV strains present in the bat samples. The use of two independent assays also helped to validate the results and minimize the chances of false negatives. Molecular analysis was performed to detect CoV RNA from bat samples. The process began with careful preparation of the samples, followed by extraction of total RNA and synthesis of cDNA. The cDNA was then screened using two different assays to identify the presence of CoV-specific genetic material. To confirm positive results, PCR products were visualized through gel electrophoresis, and selected amplicons were excised from the gel for subsequent downstream applications

3.4.1 Sample Preparation

Upon arrival at the laboratory, all collected samples were handled under sterile conditions to prevent contamination. Samples stored in VTM were first thawed on ice before further processing. Each sample was vortexed briefly to ensure homogeneity before aliquoting. A volume of 140 μ L of the VTM sample was transferred into sterile microcentrifuge tubes for further processing. Aliquots were prepared in duplicate to ensure backup samples were available for repeat testing if necessary. The remaining sample was returned to -80°C for long-term storage. To inactivate potential

High Pure Viral Nucleic Acid Extraction Kit. The mixture was then vortexed for uniform distribution and incubated at room temperature for 30 minutes to ensure complete viral lysis.

After lysis, the samples were immediately processed for RNA extraction or stored at -80°C if extraction was not performed on the same day. Strict biosafety measures, including the use of personal protective equipment (PPE) and working within a biosafety cabinet, were followed to minimize contamination risks during sample handling.

3.4.2 RNA Extraction and cDNA Synthesis

Viral RNA from bat samples was extracted with High Pure Viral Nucleic Acid Extraction Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol, and was eluted with 50 μl of Roche elution buffer. The RNA extract was kept at -80°C for further analysis. The cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) with specific primers from two established protocols, Q-assay (Quan et al., 2010) and W-assay (Watanabe et al., 2010). Briefly, 4 μl 5X RT buffer, 2 μl dNTPs (10 mM each), 1.0 μl of specific primer, 0.5 μl of Ribolock (40 U/ μl), and 1.0 μl of RevertAid Reverse Transcriptase (200 U/ μl) were mixed 5 μl of extracted RNA. Reverse transcription was performed via incubation for 10 min at 42°C , 60 min at 50°C , and held at 20°C .

3.4.3 Quan-Assay vs Watanabe-Assay for PCR Detection

The Q and W assays PCR protocols targeted the RNA-dependent polymerases (RdRp) region RNA polymerase, involving nested and hemi-nested RT-PCR. The details of primers used in this study are shown in (Table 3.1) and the illustration of the primer-targeted regions in Figure 3.2. For the Q-assay and W-assay, the PCR protocols were performed by following the published protocols (Quan et al., 2010; Watanabe et al., 2010). The PCR reaction mixture contained 5 μL of cDNA was mixed with 10 X of Taq Buffer, 3.0 mM of

MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer, 0.025 U/µL of Taq DNA Polymerase and 33.50 µL of water nuclease-free giving a final volume of 50 µL. Each assay run included a confirmed BtCoV positive control (OP328800) and a negative control (water) to validate the test. Positive control was included in each PCR assay to validate amplification and confirm assay sensitivity. This control was derived from previously sequenced BtCoV sample obtained during earlier surveillance studies in Sarawak. The identity of this control had been confirmed by Sanger sequencing, ensuring that the amplicon corresponded to the expected RdRp region of BtCoVs.

Table 3.1: List of CoV Primer Sets Utilized in This Study, Including Their Target Regions, Sequence Information, and References

Type of PCR assay	PCR format	Primer's name	Primer sequence (5' to 3')	Amplification size (bp)	Targeted region *	References
(Q)-assay	Two-step	CoV-FWD1	CGTTGGIACWAAYBTVCCWYTICARBTRGG	520	17,480-17,820	Quan et al. (2010)
	RT-PCR	CoV-RVS1	GGTCATKATAGCRTCAVMASWWGCNACATG			
	Nested	CoV-FWD2	GGCWCCWCCHGGNGARCAATT	328		
	PCR	CoV-RVS2	GGWAWCCCCAYTGYTGWAYRTC			
(W)-assay	Two-step	CoV-FWD3	GGTTGGGAYTAYCCHAARTGTGA	440	14,370-14,750	Modified from Watanabe et al. (2010)
	RT-PCR	CoV-RVS3	CCATCATCASWYRAATCATCATA			
	Hemi-nested	CoV-FWD4	GAYTAYCCHAARTGTGAYAGAGC			

*The position of PCR product was aligned and compared with the whole genome of HCoV-229E (Gen Bank accession No.: NC_002645.1) (Wacharapluesadee et al., 2024)

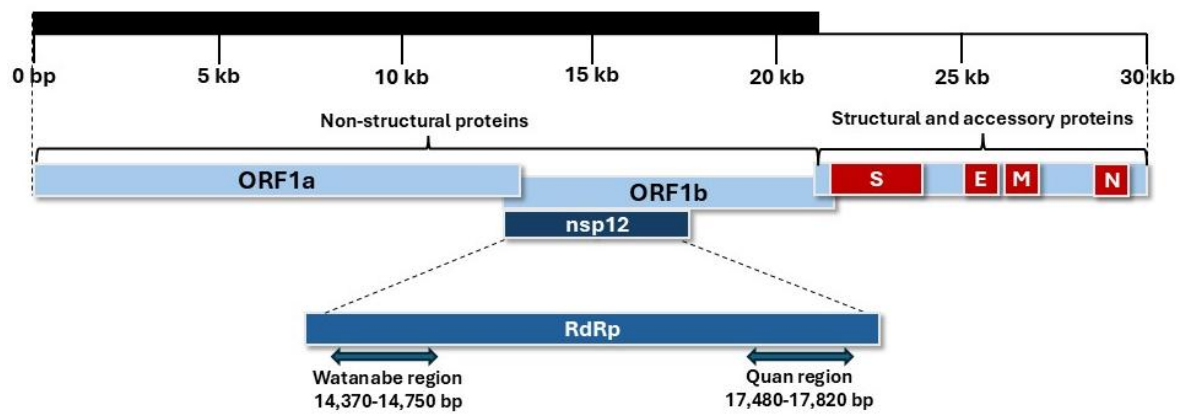


Figure 3.2: Genomic Organization of a CoV Highlighting the RdRp Region Targeted by Quan and Watanabe Primers

3.4.4 Gel Visualization and Gel Cutting

Following PCR amplification, gel electrophoresis was performed to visualize and confirm the presence of target amplicons before purification and sequencing. This step ensured that only specific PCR products of the expected size were selected for downstream analysis. PCR products were loaded onto a 2.0% agarose gel prepared with Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide (EtBr). A 100 bp DNA ladder was included alongside the samples for size reference. Electrophoresis was conducted at 120 V for 40 minutes to allow adequate separation of DNA fragments. Following electrophoresis, the gel was placed on a UV transilluminator or a blue-light imaging system to visualize the bands. Only well-defined bands corresponding to the expected amplicon size were selected for further processing. Smearing or unexpected bands were noted as potential indicators of nonspecific amplification or degraded DNA.

The target bands were carefully excised from the gel using a sterile scalpel under minimal UV exposure to prevent DNA damage. Each gel slice was placed in a sterile microcentrifuge tube for subsequent purification. To prevent cross-contamination, separate tools were used for different samples, and gloves were changed between cutting different gel bands. The excised DNA fragments were then processed for purification to remove agarose, primers, and other contaminants before sequencing. This step ensured that only high-quality, specific PCR products were used for further analysis, improving the accuracy of sequencing and downstream phylogenetic assessments.

3.4.5 Gel Purification and Amplicon Preparation for Sequencing

Following PCR amplification, gel purification was performed to isolate the target DNA fragments for sequencing. This step ensured the removal of non-specific products,

primer dimers, and potential contaminants, improving the quality of downstream sequencing data. Gel-excised DNA fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany), following the manufacturer's protocol. In brief, gel slices were solubilized in binding buffer at 50°C for 10 minutes, and the DNA was bound to a silica membrane column via centrifugation. The bound DNA was washed to remove impurities, and purified amplicons were eluted in a low-salt elution buffer or nuclease-free water.

3.4.6 Sequencing of PCR Amplicons

The purified products were subjected to Sanger sequencing to confirm the identity of the amplified fragments. Sequencing reactions were performed using the ABI® BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, USA) and run on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA) through an outsourced commercial service provider, Apical Scientific Sdn. Bhd. (Malaysia).

3.5 Bioinformatics Analysis

The bioinformatics analysis began with data preprocessing, where raw sequence data obtained from Sanger sequencing was cleaned, trimmed, and aligned using appropriate software tools to ensure high-quality sequences for downstream analysis (Lu et al., 2020). This step was crucial to eliminate poor-quality reads and sequencing artifacts, which could compromise the accuracy of the results. Subsequently, phylogenetic analysis was performed to determine the evolutionary relationships between the detected CoVs sequences and reference strains retrieved from GenBank (Liò & Goldman, 2004). Multiple sequence alignments were generated, and phylogenetic trees were constructed using Maximum Likelihood (ML) method with bootstrapping to assess tree robustness (Shaibu et al., 2021).

This analysis provided insights into the diversity and classification of the detected CoVs and helped to determine their genetic relatedness to known strains.

3.5.1 Data Preprocessing

All collected samples were assigned unique identifiers, which were cross verified against field data sheets to prevent mismatches. Sample metadata, including bat species, sampling location, collection date, and sample type, were compiled into a structured database using Microsoft Excel (version 2503) for easy retrieval and analysis. Prior to statistical and phylogenetic analysis, raw data from laboratory experiments and sequencing outputs underwent preprocessing to ensure accuracy, consistency, and reliability. This step involved data cleaning, transformation, and quality control checks to remove errors and standardize formats for further analysis. Raw sequencing reads were examined for quality using MEGA11: Molecular Evolutionary Genetics Analysis version 11.0.13 (Tamura et al., 2021). Poor-quality reads with ambiguous bases, short sequences, or sequencing artifacts were removed. In cases where sequencing errors were detected, chromatograms were manually inspected and corrected before downstream analysis. High-quality sequences were converted into FASTA format and aligned using MUSCLE in MEGA software. Ambiguous base calls were resolved, and sequences shorter than the target region were excluded from further analysis. Any discrepancies in sequence length or unexpected mutations were flagged for re-examination.

3.5.2 Phylogenetic Analysis

The identities and similarities of the sequenced isolated were analyzed using the Basic Local Alignment Search Tool (BLASTn) of the National Center for Biotechnology Information (NCBI) GenBank database. The positive CoV sequences from the Q and W

assays were aligned with reference sequences available from GenBank using MUSCLE (Edgar, 2004) (Appendix H and Appendix I). To infer evolutionary relationships, phylogenetic trees were constructed using the ML method in MEGA. The best-fitting nucleotide substitution model was determined using the Akaike Information Criterion (AIC) as AIC considers both the goodness of fit of a model and the number of parameters it uses (Tamura et al., 2021). To assess the robustness of the phylogenetic tree, 1,000 bootstrap replicates were performed. Reference sequences of known BtCoVs and related HCoVs were included to contextualize the detected viruses within the broader CoV phylogeny. For both alignments, the general time reversible (GTR) model with a gamma distribution of rate heterogeneity was the best-fitting model.

Pairwise genetic distances between sequences were calculated using the Kimura-2-Parameter model in MEGA to assess genetic divergence among detected CoVs. The dataset used in this study included 45 positive BtCoV sequences detected using the Q-assay and 31 sequences from the W-assay. Additionally, reference sequences for comparative analysis were retrieved from the GenBank database.

3.6 Cohen's Kappa (κ) and McNemar Test

Statistical analyses were conducted using IBM SPSS Statistics version 29.0.2.0 (20) software. The results from the Q-assay and W-assay were evaluated using Cohen's Kappa (κ) statistics to assess the level of agreement between the two tests. Kappa values were interpreted as follows: 0.81–1.00 indicated almost perfect agreement, 0.61–0.80 good agreement, 0.41–0.60 moderate agreement, 0.21–0.40 fair agreement, 0.00–0.20 minimal agreement, and less than 0.00 signified poor agreement between the assays (Landis & Koch,

1977). McNemar analyses the discordant pairs in the CoV test results between the Q- and W- assays. Statistical significance was set at $p < 0.05$.

3.7 Species Richness and Shannon Diversity (H')

Species richness and Shannon diversity index (H') were calculated to assess bat community diversity at the study sites. Analyses were conducted using the R package *vegan* (Oksanen et al., 2015). For each site and species group, species richness was determined as the total number of species recorded, while Shannon diversity (H') accounted for both species abundance and evenness. Data visualization, including diversity plots and heatmaps, was performed in R using *ggplot2* (Wickham, 2011) and *pheatmap* (Kolde, 2025).

3.8 Heatmap Construction

To visualize the prevalence of BtCoVs across different bat species and locations, a heatmap was constructed using the R package ‘*ggplot*’ v3.5.1 (Wickham, 2011). The dataset consisted of CoV screening results, with bat species on the x-axis and PCR assay/locality/CoV subgenus on the y-axis. The heatmap was generated by mapping prevalence rates to a red gradient scale, where darker shades indicated higher positivity rates. Additionally, host numbers were represented as red circles, with size proportional to the number of sampled individuals per species.

Data preprocessing involved structuring the dataset into a tidy format, ensuring that each observation corresponded to a species, location, and CoV subgenus with associated prevalence values. Custom colour scales and annotations were applied to enhance readability. This visualization effectively highlights patterns in CoV distribution among bat hosts and geographic regions.

CHAPTER 4

RESULTS

4.1 Bat Sample Collection

Between 2021 and 2023, we captured 346 bats in northern and western Sarawak. We collected 237 rectal swabs and 109 fecal samples from these bats, which represented 29 different species across 13 genera and 6 families (Appendix E). Most of the samples came from bats in the Pteropodidae (frugivorous bats) and Rhinolophidae (horseshoe bats) families, accounting for approximately 74.28% of the total. The most frequently sampled species were *Cynopterus brachyotis* (n = 62) and *Rhinolophus creaghi* (n = 55). This outcome reflects the effectiveness of different trapping methods: harp traps are highly efficient for capturing echolocating insectivorous bats such as *Rhinolophus* species, while mist nets are more suitable for frugivorous bats like *Cynopterus* species. For a more balanced representation, incorporating more mist nets alongside harp traps is recommended, as this combination would allow effective sampling of both groups, thereby reducing sampling bias and increasing species diversity in the dataset.

Additionally, some bat species exhibited trap avoidance behavior, reducing the chances of obtaining sufficient samples for those species. This was supported by acoustic observations using the EchoMeter, which recorded frequent echolocation calls of *Hipposideros* in areas where few or no individuals were captured. Such discrepancies between acoustic detections and physical captures suggest that certain taxa actively avoid mist nets or harp traps, leading to potential underrepresentation in the dataset. Notably, the study also included single samples from rarer species such as *Kerivoula minuta*, *Kerivoula pellucida*, *Rhinolophus luctus*, and *Rhinolophus trifolius*. This research marks the first

documented detection of BtCoVs using live bat samples in Sarawak. The detailed sampling locations are shown in Figure 4.1 and the overall bat captured during this study is listed in Table 4.1.

4.1.1 Western Region

In the western region of Sarawak, a total of 142 bats were captured, with the majority belonging to the Pteropodidae family, representing seven species. The most frequently sampled species was *C. brachyotis* (n = 53), a highly adaptable frugivorous bat commonly found in disturbed habitats such as agricultural areas, secondary forests, and urban green spaces. Other notable frugivorous bat species captured include *Balionycteris maculata* (n = 34) and *Penthetor lucasi* (n = 17). *B. maculata*, also known as the spotted-winged frugivorous bat, is typically associated with primary and secondary forests, while *P. lucasi* is often found in cave systems and forested regions. In addition to frugivorous bats, two species of insectivorous bats from the Vespertilionidae family were recorded: *Kerivoula hardwickii* (n = 1) and *Kerivoula papillosa* (n = 1). Bats from the *Kerivoula* genus, commonly known as woolly bats, are typically found in forested habitats, where they roost in foliage or tree hollows. Similarly, only one species from the Rhinolophidae family (horseshoe bats) was detected: *Rhinolophus luctus* (n = 1). *R. luctus*, also known as the great woolly horseshoe bat, is a cave-roosting species that prefers dense forest environments.

4.1.2 Niah NP

In the Niah region, 50 bats representing 15 species across 6 different families were captured. Three species each from the families Pteropodidae (*B. maculata*, *C. brachyotis*, *P. lucasi*), Hipposideridae (*Hipposideros ater*, *H. cervinus*, *H. diadema*), and Rhinolophidae (*Rhinolophus borneensis*, *Rhinolophus philippinensis*, *R. trifolius*) were present. One

species was identified from each of the families Miniopteridae (*Miniopterus magnater*) and Nycteridae (*Nycteris tragata*). The species composition was more diverse, with no single species overwhelmingly dominant. The most frequently sampled species included *R. creaghi* (n = 8) and *C. brachyotis* (n = 7). Moreover, the capture of three rare *Myotis gomantongensis* specimens was a significant find in the region. The caves and forested areas of Niah NP are known to support a high diversity of bat species, particularly those that rely on caves for roosting (Stimpson, 2012).

4.1.3 Mulu NP

Mulu had the highest bat diversity among the regions sampled, with 154 individuals representing 16 species. *Rhinolophus creaghi* (n=55) and *Hipposideros galeritus* (n=30) were the most common. Six *Hipposideros* species were recorded: *H. bicolor* (n=2), *H. cervinus* (n=18), *H. coxi* (n=2), *H. diadema* (n=3), *H. dyacorum* (n=2), and *H. galeritus* (n=30). Mulu was the only location where *H. bicolor*, *H. coxi*, *H. dyacorum*, and *H. galeritus* were found. Similarly, the only records of *Kerivoula minuta* and *Murina* sp. were from this site. This exceptional bat diversity highlights Mulu's ecological importance as a unique habitat for a variety of bat species, further emphasizing its role as a key biodiversity conservation.

Table 4.1: Bat Species Recorded in the Western Region, Niah NP, and Mulu NP, Sarawak. The Table Provides Details on Family, Species, Number of Individuals Sampled, with Their Associated Habitat and Ecological Characteristics

Bat Species	Habitat and Ecology (Based on data published in IUCN website)	Locality			Total
		Western	Niah NP	Mulu NP	
<u>Family Pteropodidae</u>					
<i>Balionycteris maculata</i>	Forest, artificial/terrestrial	17	3	1	21
<i>Cynopterus brachyotis</i>		53	7	2	62
<i>Macroglossus minimus</i>		12	-	-	12
<i>Cynopterus horsfieldi</i>	Forest, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	6	-	-	6
<i>Eonycteris spelaea</i>		9	-	-	9
<i>Dycopterus spadiceus</i>	Forest, caves and Subterranean habitats (non-aquatic)	8	-	-	8
<i>Penthetor lucasi</i>		34	2	-	36
<u>Family Hipposideridae</u>					
<i>Hipposideros ater</i>	Forest, savanna, shrubland, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	2	-	2

Table 4.1 continued

<i>Hipposideros bicolor</i>	Forest, caves, Subterranean habitats (non-aquatic)	-	-	2	2
<i>Hipposideros dyacorum</i>		-	-	2	2
<i>Hipposideros cervinus</i>	Forest, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	5	18	23
<i>Hipposideros coxi</i>	Caves, forested understorey	-	-	2	2
<i>Hipposideros diadema</i>	Forest, savanna, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	2	3	5
<i>Hipposideros galeritus</i>	Forest, rocky areas (eg. Inland cliffs, mountain peaks), caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	-	30	30
<u>Family Vespertilionidae</u>					
<i>Kerivoula hardwicki</i>	Forest, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	1	-	1	2

Table 4.1 continued

<i>Kerivoula minuta</i>	Understorey of dipterocarp forest, lowland and secondary forest	-	-	1	1
<i>Kerivoula papillosa</i>	Lowland mixed-deciduous dipterocarp forest	1	-	1	2
<i>Kerivoula pellucida</i>	Primary forest, limestone forest areas	-	1	-	1
<i>Murina suilla</i>	Forest, artificial/terrestrial	-	2	-	2
<i>Murina sp.</i>		-	-	2	2
<i>Myotis horsfieldi</i>	Forest, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	3	-	3
<i>Myotis gomantongensis</i>	Caves and Subterranean habitats (non-aquatic)	-	3	-	3
<u>Family Miniopteridae</u>					
<i>Miniopterus magnater</i>	Forest, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	4	-	4
<u>Family Nycteridae</u>					

Table 4.1 continued

<i>Nycteris tragata</i>	Forest, rocky areas (eg. Inland cliffs, mountain peaks), caves, Subterranean habitats (non-aquatic),	-	1	2	3
<u>Family Rhinolophidae</u>					
<i>Rhinolophus borneensis</i>	Forest, rocky areas (eg. Inland cliffs, mountain peaks), caves, Subterranean habitats (non-aquatic),	-	8	20	28
<i>Rhinolophus luctus</i>		1	-	-	1
<i>Rhinolophus creaghi</i>	Forest, caves, Subterranean habitats (non-aquatic)	-	-	55	55
<i>Rhinolophus philippinensis</i>	Forest, caves, Subterranean habitats (non-aquatic), artificial/terrestrial	-	6	12	18
<i>Rhinolophus trifolius</i>	Forest, artificial/terrestrial	-	1	-	1
Total		142	50	154	346

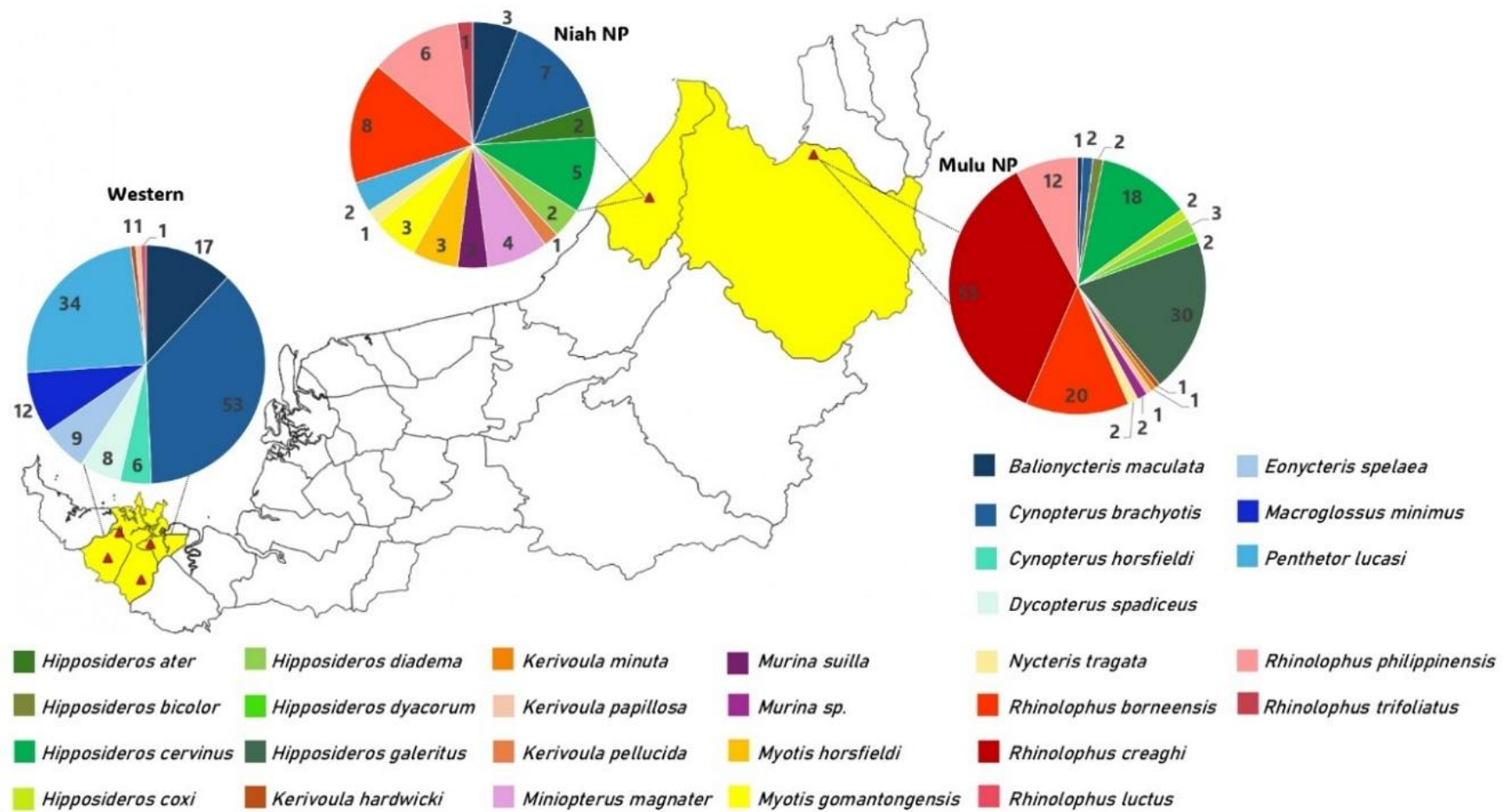


Figure 4.1: Geographic distribution and bat species diversity in northern and western Sarawak, Malaysian Borneo. Pie charts represent the relative abundance of bat species sampled from three regions: Western Sarawak, Niah NP, and Mulu NP. The chart segments are color-coded to indicate different bat species, with species names listed in the legend. Sampling sites are marked on the map with red triangles.

4.1.4 Species Richness and Shannon Index (H')

We assessed bat community diversity at the three study sites using species richness and the Shannon diversity index (H') (Figure 4.2). Species richness was highest at Mulu National Park (16 species), followed by Niah National Park (15 species) and the western region (10 species). Shannon diversity (H') was highest at Niah National Park ($H' = 2.511$), indicating a more even distribution of individuals among species, compared to Mulu NP ($H' = 1.947$) and the western region ($H' = 1.748$). These results suggest that while Mulu NP had slightly higher species richness, Niah NP supported a more evenly distributed bat community.

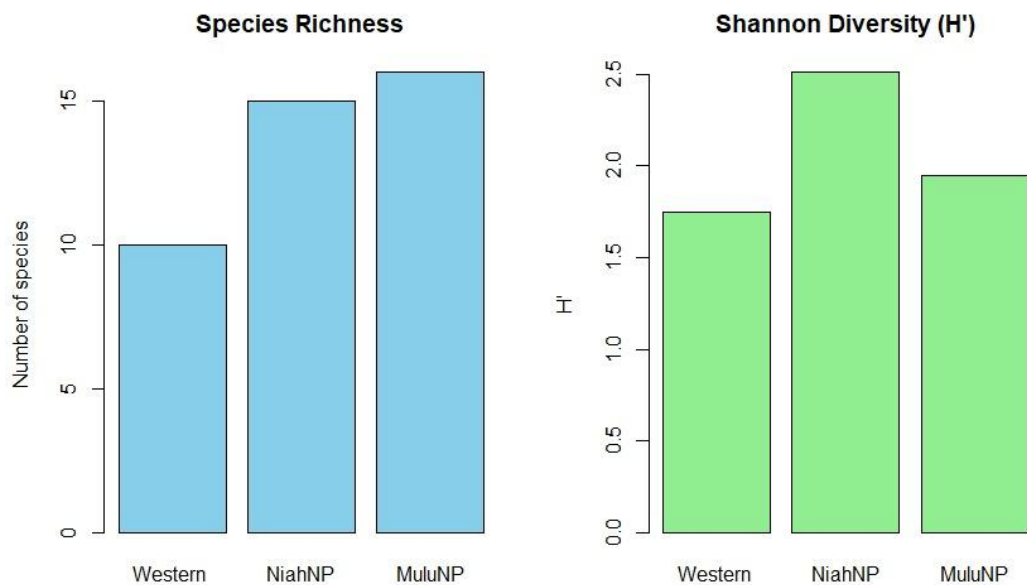


Figure 4.2: Barplot showing species richness (blue) and Shannon diversity index (H') (green) of bats across the three study regions (Western, Niah NP and Mulu NP)

A heatmap of species abundance across sites was generated using pheatmap, visually highlighting differences in species composition and relative abundance among sites. The heatmap further illustrates that some species were restricted to specific sites, while others were more widely distributed, providing an intuitive overview of bat community structure in the study regions (Figure 4.3). The western region was more concentrated with frugivorous bats, Niah NP showed a more even distribution across species, and Mulu NP was dominated by *Rhinolophus* and *Hipposideros* species. These patterns provide an intuitive overview of bat community structure and potential ecological contexts for BtCoV prevalence.

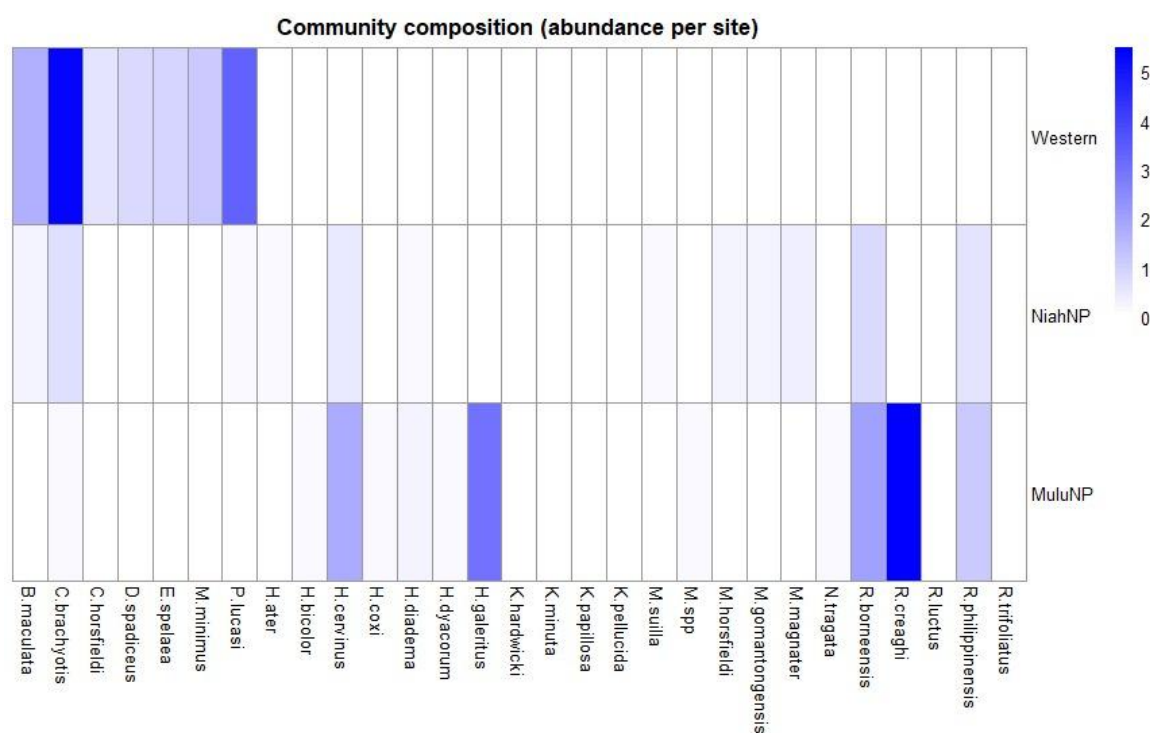


Figure 4.3: Heatmap showing the relative abundance of 29 bat species across the three study regions (Western, Niah NP and Mulu NP). The color intensity represents species abundance, with darker shades indicating higher numbers of individuals

4.2 BtCoV Detection

In this section, the detection of BtCoV positivity is presented through multiple approaches. Both conventional PCR and confirmatory sequencing were applied to ensure the accuracy of detection. The results are further organized by bat species, sampling localities, and CoV clades to provide ecological and phylogenetic context. In addition, comparisons between the Q-assay and W-assay protocols are highlighted to evaluate their relative sensitivity and reliability in detecting diverse BtCoV lineages.

4.2.1 BtCoV Detection Across Species and Localities

Firstly, we analyzed the distribution BtCoVs across different bat species and localities, namely the western region, Niah NP, and Mulu NP (Table 4.2). The overall BtCoV positivity rate across all locations was 22.83% (79/346), with variations observed between species and regions. BtCoV were found in a diverse range of bat species (13 in total) across four families in western and northern Sarawak (Figure 4.4). Among frugivorous bats, *C. brachyotis* had the highest prevalence (37.10%, 23/62), making it one of the most significant carriers of BtCoVs in the western region, followed by *P. lucasi* and *E. spelaea* (both at 33.33% positivity). Insectivorous bats showed varying rates, with *R. creaghi* having the highest positivity rate with 24 out of 55 individuals (43.63%), primarily from Mulu NP, while *R. borneensis* and *R. philippinensis* had lower rates. Only one *Miniopterus magnater* tested positive (25%). On the other hand, several bat species, including *C. horsfieldi*, *H. ater*, *H. bicolor*, and multiple species from the genera *Kerivoula* and *Myotis*, recorded zero positivity across all locations, indicating either a low prevalence or absence of detectable BtCoVs in these species. Areas in Sarawak with higher bat populations, as determined by the number of individuals captured during sampling, also showed a greater likelihood of BtCoV detection. Although bats were not permanently marked, the use of nail colouring as

a temporary marker reduced the risk of duplicate sampling. This approach is a commonly used, non-invasive field technique that provides short-term individual recognition without harming the animals.

Table 4.2: BtCoV Positivity Based on Bat Species and Sampling Localities in Sarawak. The Table Shows the Number of Individuals Captured and the Number Testing Positive at Each Site

Species	Number of individuals	BtCoV Positivity			
		Western	Niah NP	Mulu NP	Proportion of bats tested positive (%)
<i>Balionycteris maculata</i>	21	1	0	0	$1/21 = 4.76$
<i>Cynopterus brachyotis</i>	62	22	1	0	$23/62 = 37.10$
<i>Cynopterus horsfieldi</i>	6	0	0	0	0
<i>Dycopterus spadiceus</i>	8	2	0	0	$2/8 = 0.25$
<i>Eonycteris spelaea</i>	9	3	0	0	$3/9 = 33.33$
<i>Macroglossus minimus</i>	12	1	0	0	$1/12 = 8.33$
<i>Penthetor lucasi</i>	36	12	0	0	$12/36 = 33.33$
<i>Hipposideros ater</i>	2	0	0	0	0
<i>Hipposideros bicolor</i>	2	0	0	0	0
<i>Hipposideros cervinus</i>	23	0	0	2	$2/23 = 8.70$
<i>Hipposideros coxi</i>	2	0	0	0	0
<i>Hipposideros diadema</i>	5	0	0	0	0
<i>Hipposideros dyacorum</i>	2	0	0	0	0
<i>Hipposideros galeritus</i>	30	0	0	1	$1/30 = 3.33$
<i>Kerivoula hardwicki</i>	2	0	0	0	0
<i>Kerivoula minuta</i>	1	0	0	0	0

Table 4.2 continued

<i>Kerivoula papillosa</i>	2	0	0	0	0
<i>Kerivoula pellucida</i>	1	0	0	0	0
<i>Murina suilla</i>	2	0	0	0	0
<i>Murina sp.</i>	2	0	0	0	0
<i>Myotis horsfieldi</i>	3	0	0	0	0
<i>Myotis gomantongensis</i>	3	0	0	0	0
<i>Miniopterus magnater</i>	4	0	1	0	$1/4 = 0.25$
<i>Nycteris tragata</i>	3	0	0	0	0
<i>Rhinolophus borneensis</i>	28	0	0	4	$4/28 = 14.29$
<i>Rhinolophus creaghi</i>	55	0	0	24	$24/55 = 43.67$
<i>Rhinolophus luctus</i>	1	1	0	0	$1/1 = 100$
<i>Rhinolophus philippinensis</i>	18	0	0	4	$4/18 = 22.22$
<i>Rhinolophus trifolius</i>	1	0	0	0	0
Total	346	42	2	35	$79/349 = 22.83$

4.2.2 BtCoV Detection using Two Molecular Assays

Next, we compare BtCoV detection using two molecular assays, Q and W, along with their overlap (Q&W) (Table 4.3). The highest number of positive detections was recorded in *R. creaghi*, with 21 cases detected by the Q assay, 10 by the W assay, and 7 testing positive in both assays (Q&W), making it the species with the highest overall CoV positivity. *C. brachyotis* also exhibited a high detection rate, with 11 positive cases in Q, 17 in W, and 8 in Q&W, suggesting a substantial BtCoV presence. Conversely, several species,

including *C. horsfieldi*, *H. ater*, *H. bicolor*, *M. horsfieldi*, and *M. gomantongensis*, had no positive detections in either assay, further reinforcing their potential low susceptibility to BtCoVs or undetectable viral loads in this study. The detection discrepancy between Q and W assays highlights the importance of using multiple assays for comprehensive virus identification.

Table 4.3: BtCoV Positivity Based on Bat Species and Assays (Q-Assay and W-Assay). The Table Presents the Number of Individuals Captured and the Number Testing Positive in Each Assay

Species	Number of individuals	BtCoV Positivity		
		Q	W	Q&W
<i>Balionycteris maculata</i>	21	0	1	0
<i>Cynopterus brachyotis</i>	62	11	17	8
<i>Cynopterus horsfieldi</i>	6	0	0	0
<i>Dycopterus spadiceus</i>	8	0	2	0
<i>Eonycteris spelaea</i>	9	1	2	0
<i>Macroglossus minimus</i>	12	1	0	0
<i>Penthetor lucasi</i>	36	4	9	1
<i>Hipposideros ater</i>	2	0	0	0
<i>Hipposideros bicolor</i>	2	0	0	0
<i>Hipposideros cervinus</i>	23	2	1	1
<i>Hipposideros coxi</i>	2	0	0	0
<i>Hipposideros diadema</i>	5	0	0	0
<i>Hipposideros dyacorum</i>	2	0	0	0
<i>Hipposideros galeritus</i>	30	1	0	0
<i>Kerivoula hardwicki</i>	2	0	0	0

Table 4.3 continued

<i>Kerivoula minuta</i>	1	0	0	0
<i>Kerivoula papillosa</i>	2	0	0	0
<i>Kerivoula pellucida</i>	1	0	0	0
<i>Murina suilla</i>	2	0	0	0
<i>Murina sp.</i>	2	0	0	0
<i>Myotis horsfieldi</i>	3	0	0	0
<i>Myotis gomantongensis</i>	3	0	0	0
<i>Miniopterus magnater</i>	4	1	1	1
<i>Nycteris tragata</i>	3	0	0	0
<i>Rhinolophus borneensis</i>	28	4	0	0
<i>Rhinolophus creaghi</i>	55	21	10	7
<i>Rhinolophus luctus</i>	1	0	1	0
<i>Rhinolophus philippinensis</i>	18	4	0	0
<i>Rhinolophus trifoliatus</i>	1	0	0	0
Total	346	50	44	18

4.2.3 BtCoV Detection Based on CoV Subgenus

Another approach that was used in illustrating BtCoV positivity was analyzing the data based on their CoV subgenus, helping to identify which bat species serve as reservoirs for specific CoV lineages (Table 4.4). The highest diversity of CoV subgenera was again found in *R. creaghi*, which harbored four different CoV subgenera (*Rhinovirus-rCoV*, *Unclassified Borneo- α -CoV*, *Nobecovirus-rCoV*, and *Sarbecovirus-rCoV*), demonstrating its

role as a significant host for multiple CoVs. *C. brachyotis* was notably associated with *Nobecovirus-rCoV* (15 cases). Meanwhile, *M. magnater* was the only species in which *Minunacovirus-rCoV* was detected, reinforcing its unique role as a reservoir for this subgenus. Among the *Hipposideros* species, only *H. cervinus* and *H. galeritus* tested positive for BtCoVs, with a total of three positive individuals. *H. cervinus* had two positive individuals, each associated with different CoV subgenera, one individual carried *Unclassified Borneo- α -CoV* and another one individual carried *Sarbecovirus-rCoV*. Whereas *H. galeritus* had one positive individual, which was associated with *Nobecovirus-rCoV*. The lowest overall CoV subgenus diversity was observed in multiple species that tested negative across all subgenera, including *B. maculata*, *H. ater*, *H. bicolor*, and *M. gomantongensis*. The overall positivity is summarized in Table 4.5.

Table 4.4: BtCoV Positivity Based on Bat Species and Detected CoV Subgenera. The Table Shows the Number of Individuals Captured and the Number Testing Positive Within Each Subgenus

Species	Number of individuals	BtCoV Positivity					
		Minunacovirus-rCoV	Rhinovirus-rCoV	Unclassified Borneo- α -CoV	Nobecovirus-rCoV	Sarbecovirus-rCoV	Total
<i>Balionycteris maculata</i>	21	0	0	0	0	0	0
<i>Cynopterus brachyotis</i>	62	0	0	3	15	0	18
<i>Cynopterus horsfieldi</i>	6	0	0	0	0	0	0
<i>Dycopterus spadiceus</i>	8	0	0	2	0	0	2
<i>Eonycteris spelaea</i>	9	0	0	0	1	0	1
<i>Macroglossus minimus</i>	12	0	0	0	1	0	1
<i>Penthetor lucasi</i>	36	0	0	0	6	0	6

Table 4.4 continued

<i>Hipposideros ater</i>	2	0	0	0	0	0	0
<i>Hipposideros bicolor</i>	2	0	0	0	0	0	0
<i>Hipposideros cervinus</i>	23	0	0	1	0	1	2
<i>Hipposideros coxi</i>	2	0	0	0	0	0	0
<i>Hipposideros diadema</i>	5	0	0	0	0	0	0
<i>Hipposideros dyacorum</i>	2	0	0	0	0	0	0
<i>Hipposideros galeritus</i>	30	0	0	0	1	0	1
<i>Kerivoula hardwicki</i>	2	0	0	0	0	0	0

Table 4.4 continued

<i>Kerivoula minuta</i>	1	0	0	0	0	0	0
<i>Kerivoula papillosa</i>	2	0	0	0	0	0	0
<i>Kerivoula pellucida</i>	1	0	0	0	0	0	0
<i>Murina suilla</i>	2	0	0	0	0	0	0
<i>Murina sp.</i>	2	0	0	0	0	0	0
<i>Myotis horsfieldi</i>	3	0	0	0	0	0	0
<i>Myotis gomantongensis</i>	3	0	0	0	0	0	0
<i>Miniopterus magnater</i>	4	1	0	0	0	0	1
<i>Nycteris tragata</i>	3	0	0	0	0	0	0
<i>Rhinolophus borneensis</i>	28	0	0	4	0	0	4

Table 4.4 continued

<i>Rhinolophus creaghi</i>	55	0	4	20	1	3	28
<i>Rhinolophus luctus</i>	1	0	0	1	0	0	1
<i>Rhinolophus philippinensis</i>	18	0	0	3	0	0	3
<i>Rhinolophus trifolius</i>	1	0	0	0	0	0	0
Total	346	1	4	34	25	4	68

Table 4.5: Results of Q-Assay and W-Assay for the Western and Northern Regions. The Table Shows the Number of Individuals Captured, With the Number Testing Positive Indicated in Square Brackets []

Bat species	Western region			Northern region						Total
				Niah National Park			Mulu National Park			
	No. bats	Q / W	Q & W	No. bats	Q / W	Q & W	No. bats	Q / W	Q & W	
<u>Family Pteropodidae</u>										
<i>Balionycteris maculata</i>	17	1	0	3	0	0	1	0	0	21 [1]
<i>Cynopterus brachyotis</i>	53	13	9	7	1	0	2	0	0	62 [23]
<i>Cynopterus horsfieldi</i>	6	0	0	0	0	0	0	0	0	6
<i>Dycopterus spadiceus</i>	8	2	0	0	0	0	0	0	0	8 [2]
<i>Eonycteris spelaea</i>	9	3	0	0	0	0	0	0	0	9 [3]
<i>Macroglossus minimus</i>	12	1	0	0	0	0	0	0	0	12 [1]
<i>Penthetor lucasi</i>	34	12	0	2	0	0	0	0	0	36 [12]
<u>Family Hipposideridae</u>										
<i>Hipposideros ater</i>	0	0	0	2	0	0	0	0	0	2
<i>Hipposideros bicolor</i>	0	0	0	0	0	0	2	0	0	2
<i>Hipposideros cervinus</i>	0	0	0	5	0	0	18	2	0	23 [2]
<i>Hipposideros coxi</i>	0	0	0	0	0	0	2	0	0	2
<i>Hipposideros diadema</i>	0	0	0	2	0	0	3	0	0	5

Table 4.5 continued

<i>Hipposideros dyacorum</i>	0	0	0	0	0	0	2	0	0	2
<i>Hipposideros galeritus</i>	0	0	0	0	0	0	30	1	0	30 [1]
<u>Family Vespertilionidae</u>										
<i>Kerivoula hardwicki</i>	1	0	0	0	0	0	1	0	0	2
<i>Kerivoula minuta</i>	0	0	0	0	0	0	1	0	0	1
<i>Kerivoula papillosa</i>	1	0	0	0	0	0	1	0	0	2
<i>Kerivoula pellucida</i>	0	0	0	1	0	0	0	0	0	1
<i>Murina suilla</i>	0	0	0	2	0	0	0	0	0	2
<i>Murina</i> sp.	0	0	0	0	0	0	2	0	0	2
<i>Myotis horsfieldi</i>	0	0	0	3	0	0	0	0	0	3
<i>Myotis gomantongensis</i>	0	0	0	3	0	0	0	0	0	3
<u>Family Miniopteridae</u>										
<i>Miniopterus magnater</i>	0	0	0	4	0	1	0	0	0	4 [1]
<u>Family Nycteridae</u>										
<i>Nycteris tragata</i>	0	0	0	1	0	0	2	0	0	3
<u>Family Rhinolophidae</u>										
<i>Rhinolophus borneensis</i>	0	0	0	8	0	0	20	4	0	28 [4]
<i>Rhinolophus creaghi</i>	0	0	0	0	0	0	55	17	7	55 [24]

Table 4.5 continued

<i>Rhinolophus luctus</i>	1	1	0	0	0	0	0	0	0	1 [1]
<i>Rhinolophus philippinensis</i>	0	0	0	6	0	0	12	4	0	18 [4]
<i>Rhinolophus trifolius</i>	0	0	0	1	0	0	0	0	0	1
Total	142 [42]			50 [2]			154 [35]			346 [79]

*No. bats represent the number of bat individuals captured in each locality.

Q/W represents the number of bats CoV positive in Q or W assay.

Q&W represents the number of bats CoV positive in both Q and W assays.

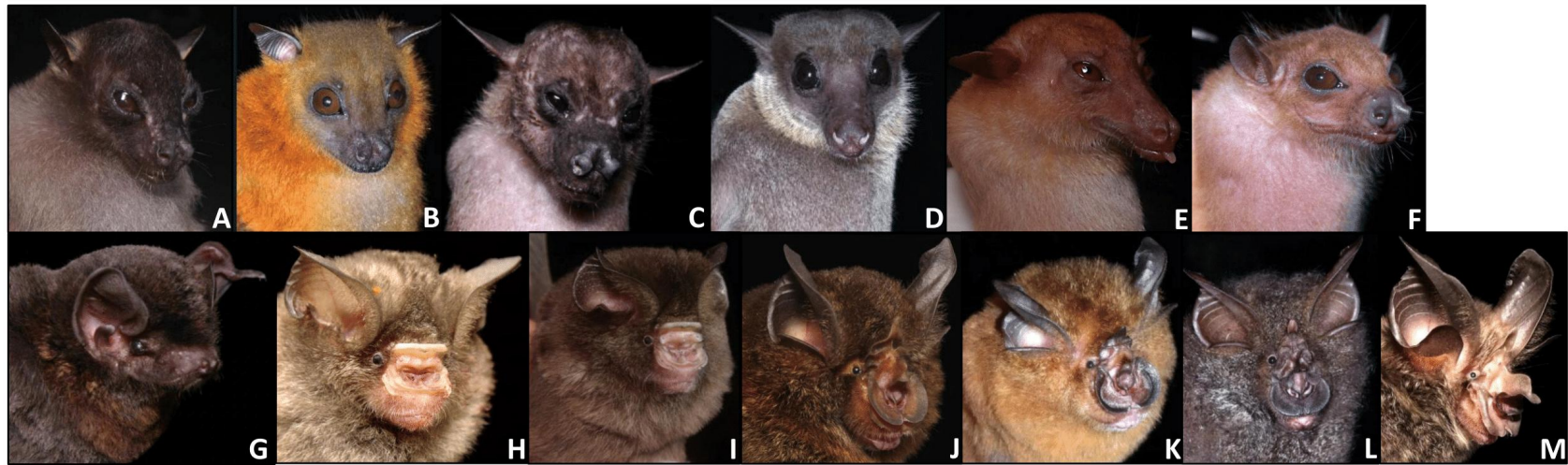


Figure 4.4: The photographs of the 13 CoV positive bat species are shown here. Family Pteropodidae: A-*Balionycteris maculata*; B-*Cynopterus brachyotis*; C-*Dycopterus spadiceus*; D-*Eonycteris spelaea*; E-*Magroglossus minimus*; F-*Penthetor lucasi*; Family Miniopteridae: G-*Miniopterus magnater*; Family Hipposideridae: H-*Hipposideros cervinus*; I-*Hipposideros galeritus*; Family Rhinolophidae: J-*Rhinolophus borneensis*; K-*Rhinolophus creaghi*; L-*Rhinolophus luctus*; M-*Rhinolophus philippinensis*

4.3 Phylogenetic Classification of BtCoVs

Phylogenetic analysis using RdRp sequences from two assays (Q-assay with 45 sequences and W-assay with 31 sequences) revealed diverse bat CoVs belonging to six subgenera (Figure 4.5). These included two *unclassified Borneo-Alpha CoVs* (*Clade A2* and *Clade A3*), and unclassified CoVs within the subgenera *Minunacovirus*, *Rhinolophus*, *Nobecovirus*, and *Sarbecovirus* (Appendix F and Appendix G). Due to the limited genetic information available from a partial fragment of the RdRp gene, definitive classification of the detected sequences according to established CoV taxonomy criteria (as defined by the ICTV) was not possible. While the RdRp region is highly conserved and suitable for preliminary detection and broad subgenus assignment, longer genomic regions or complete genome sequences are required for reliable species-level classification. *Nobecovirus*-related CoVs were predominantly found in *Pteropodidae* bats. *Rhinolophus*-related CoVs were detected in *Rhinolophidae* bats, and *Minunacovirus*-related CoVs were found in *Miniopteridae* bats.

4.3.1 *Minunacovirus-rCoVs* (Clade A1)

The *Minunacovirus-rCoVs* (*Clade A1*) were found in *Miniopterus magnater* bat sample NNP-064 from Niah National Park. This clade showed perfect congruence in both assays, also include strains from China and Hong Kong, BatCoV/MpGD17/*M.pusillus*/China/2017(OQ175069), BatCoV/AFCD307/*Miniopterus*/HongKong (EU420137), and BatCoV/HKU8/AFCD77/*Miniopterus*/Hong Kong (EU420139), exhibiting 91.99–93.20% sequence similarity. This suggests a close relationship between these viruses and supports *Miniopterus* bats as a reservoir for *Minunacovirus*.

4.3.2 *Unclassified Borneo-α CoVs (Clade A2 and Clade A3)*

Basic congruence was observed in unclassified Borneo-α CoVs (*Clade A2* and *Clade A3*) across both assays. The sequences from *Rhinolophus* species from Mulu NP, *R. borneensis*, *R. creaghi* and *R. philippinensis* prominently grouped in *Clade A2* together with other *Rhinolophus* and *Hipposideros* species from China, Jordan, and Malaysia. Notably, *Clade A2* was only observed in Q-assay and formed a sister clade to the *Pedacovirus* lineage (AF353511). In contrast, *Clade A3* displayed greater divergence than *Clade A2*, with a broader spread of sequences from both frugivorous and insectivorous bat species, including previously described BorneoAlpha-1 (MZ574065) and BorneoAlpha-2 (OP328796) (bolded in blue in Figure 4.5) indicating higher genetic variability within this group. Similarly, *Clade A3* was only observed in W-assay with no overlapping sequences with *Clade A2*. Additionally, there were unclassified Borneo-Alpha CoVs from *Clade A2* and *Clade A3*, indicating the presence of novel strains yet to be fully characterized. Interestingly, 2 concordance sequences from this study, *R. creaghi* from Mulu National Park (MNP002 and MNP023) were observed in both assays. However, these sequences group in Borneo-Alpha CoVs (*Clade A2*) in Q-assay and *Rhinocovirus-rCoVs (Clade A4)* in W-assay.

4.3.3 *Rhinocovirus-rCoVs (Clade A4)*

The *Rhinocovirus-rCoVs (Clade A4)* was observed to cluster together with SADS-rCoV/141378/*R. pusillus*/China/2014 (MF769476) and BatCoV/HKU2/*R. affinis*/China (MN312347) with sequence similarity of 83.70%, highlighting their genetic proximity.

4.3.4 *Nobecovirus-rCoVs (Clade B1)*

In the case of beta-CoVs, a distinct clade, *Nobecovirus-rCoVs (Clade B1)* was consistently identified across both assays. This clade was predominantly associated with *C.*

brachyotis and other frugivorous bats, including *P. lucasi*, *M. minumus*, and *E. spelaea*. Notably, in the Q-assay, bat CoVs from *Hipposideros* and *Rhinolophus* species were observed clustering together with those from other frugivorous bats. In this clade, six bat CoV sequences from *C. brachyotis* in this study were concordance in both assays (KPG008, KBP1002, MG023, MG055, MG065, and MG066). Overall, our bat CoV sequences in *Clade B1* were grouped together with *Nobecovirus* sequences, BatCoV/GCCDC1356/*R. leschenaulti*/China/2014 (KU762338) and BatCoV/HKU9-4/China (EF065516) as well as with other *Pteropus* species bat CoV sequences from Thailand, Cambodia and India and previously described BorneoBeta-3 (OP328793).

4.3.5 *Sarbecovirus-rCoVs* (Clade B2)

The detection of *Sarbecovirus-rCoVs* (*Clade B2*) in *R. creaghi* from Mulu NP represents one of the most significant findings of this study. The identified sequence (MNP063) clustered closely with BatCoV/PREDICTCoV84/PSW01983/*R. creaghi*/Malaysia/2016 (MT083289), showing a high sequence similarity of 97.83% in the W-assay. Moreover, it formed a sister relationship with well-characterized *Sarbecoviruses* such as BANAL-20-52 and RaTG13, lineages of particular interest due to their close evolutionary relationship with SARS-CoV-2. This provides the first molecular evidence of *Sarbecovirus*-related sequences in Sarawak, expanding their known distribution and highlighting the continued circulation of these lineages in Southeast Asian bat populations. Interestingly, the same sequence (MNP063) exhibited discordant clustering between assays: grouping within the Alpha-CoV genus (*Clade A2*) in the Q-assay but within the Beta-CoV genus (*Clade B2*) in the W-assay. In the Q-assay, this sequence clustered with BatCoV/PREDICT CoV-78/PSW01471/*R. cf. arcuatus*/Malaysia/2015 (MT064634) with sequence similarity of 92.51%.

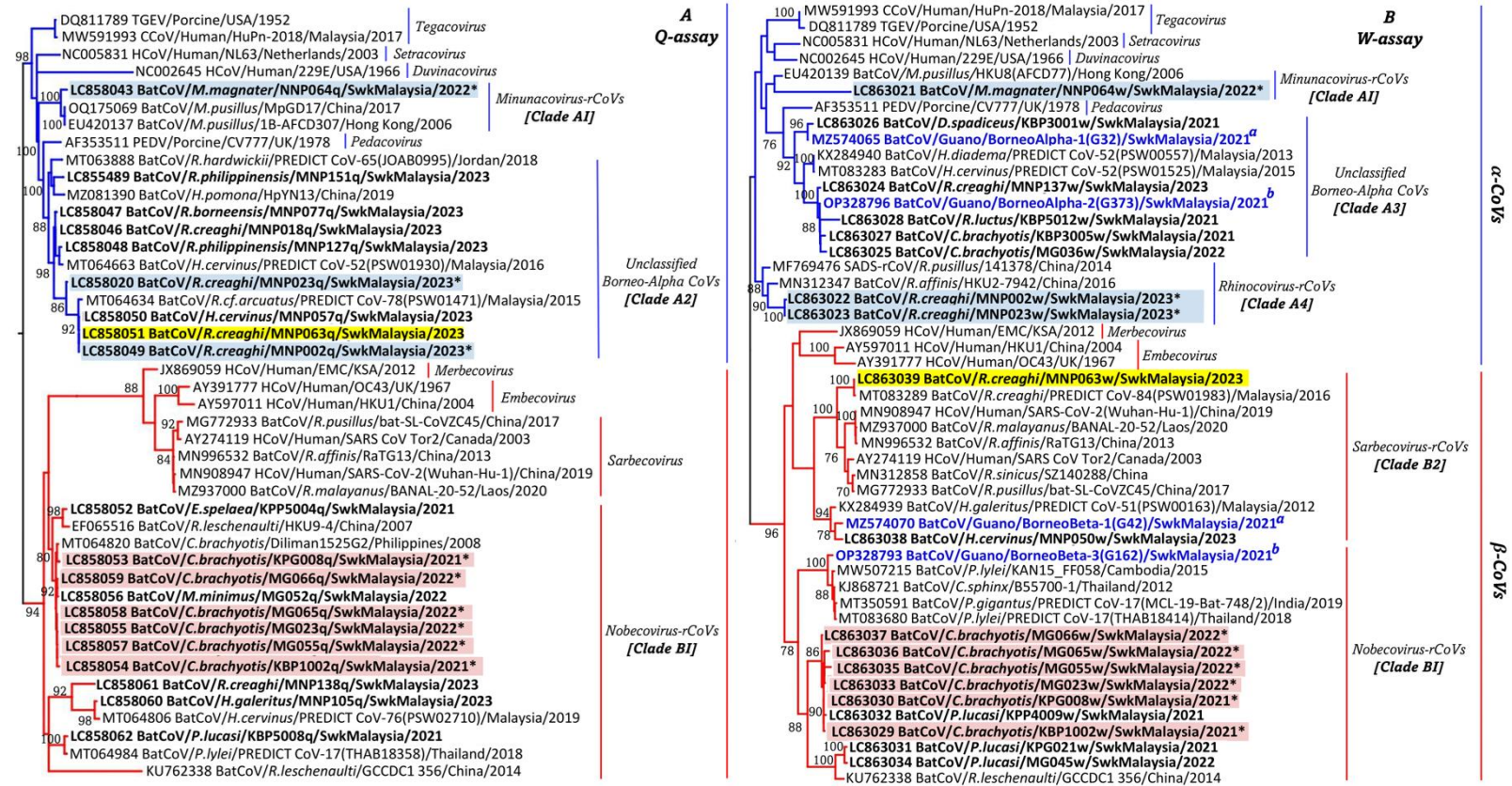


Figure 4.5: Maximum likelihood phylogenetic tree for representative partial bat CoV RdRp sequences detected using both Quan (Panel A) and Watanabe (Panel B) assays. The trees represent the evolutionary relationships of Alpha-CoVs (blue branches) and Beta-CoVs (red branches), with bootstrap values indicated at branch nodes (values $\geq 70\%$). Black bolded sequences indicate those detected in this study. Blue bolded sequences with superscripts represent representative sequences from previous studies: *a*: Tan et al., 2021, and *b*: Habeebur-Rahman et al., 2023. Sequences highlighted in blue indicate concordance within Alpha-CoVs, while sequences highlighted in red indicate concordance within Beta-CoVs. Sequences highlighted in yellow represent discordant sequence detected by the Quan and Watanabe assays

4.4 Concordance between the Q-Assay and W-Assay

The contingency Table 4.6 shows the detection of CoVs in frugivorous and insectivorous bats using two assays (Q and W). Of the total 346 samples, 18 samples were positive for both assays (W+Q+) and 270 samples were negative for both assays (W-Q-). The Kappa statistic indicates a fair agreement between the two assays (Quan and Watanabe), with a Kappa value of 0.286, which is statistically significant ($p < 0.001$). This suggests that while there is some level of agreement, the classification between the two assays is not perfect. The McNemar Test result showed a p-value of 0.512, suggesting no significant difference between the two assays (Quan and Watanabe) in terms of their performance in detecting bat CoVs (as $p > 0.05$).

The rationale for distinguishing frugivorous and insectivorous bats is based on their differing ecological roles, behaviors, and associations with viral reservoirs. Frugivorous bats (Pteropodidae) are non-echolocating, forage over wide ranges, and are recognized reservoirs for several zoonotic viruses, whereas insectivorous bats (e.g., Rhinolophidae, Hipposideridae) use echolocation, roost in caves or forest interiors, and are strongly associated with SARS-related CoVs. Presenting the results separately for these two groups allows for a better understanding of how ecological differences may influence BtCoV prevalence and diversity.

Table 4.6: Contingency Table of Q-Assay vs W-Assay Results for BtCoV Detection in Frugivorous and Insectivorous Bats. The “+” Symbol Indicates Positive Detection, While the “-” Symbol Indicates Negative Detection

Test A	W+	W-	Total
Frugivorous & Insectivorous bats			
Q+	18	32	50
Q-	26	270	296
Total	44	302	346
Test B	W+	W-	Total
Frugivorous bats			
Q+	9	8	17
Q-	22	115	137
Total	31	123	154
Test C	W+	W-	Total
Insectivorous bats			
Q+	9	24	33
Q-	4	155	159
Total	13	179	192

4.5 Heatmap Analysis

The heatmap illustrates the detection of BtCoVs across different bat families, genera, and species, as well as their prevalence in three sampling locations in Sarawak: Western, Miri, and Niah (Figure 4.6). The x-axis lists the sampled bat species, while the y-axis categorizes BtCoVs into different subgenera, including *Sarbecovirus-rCoV*, *Nobecovirus-rCoV*, *unclassified Borneo-α-CoV*, *Rhinovirus-rCoV*, and *Minunacovirus-rCoV*. The

intensity of color represents the level of BtCoV detection, with darker shades indicating higher positivity rates. The host number, represented by red dots at the bottom of the heatmap, indicates the number of individuals tested per species, with larger dots corresponding to a greater number of samples. This visual representation helps contextualize the detection rates in relation to the number of bats tested.

Among the bat species tested, *C. brachyotis* and *P. lucasi* (Pteropodidae) showed strong detection signals for *Nobecovirus-rCoV*. Some samples within this bat family were also identified as *unclassified Borneo-α-CoV*. Whereas the genus *Rhinolophus* were identified in all the CoV subgenera except for *Minunacovirus-rCoV*. Notably, *R. creaghi* were detected for *Rhinovirus-rCoV* and *Sarbecovirus-rCoV*. These results indicate that BtCoVs were detected across multiple families, with varying levels of positivity. The sole representative from Miniopteridae, *M. magnater*, tested positive for *Minunacovirus-rCoV*.

The distribution of BtCoV-positive samples across the three sampling locations shows that the western region of Sarawak and Mulu NP had the highest frequency of BtCoV detections. In particular, *R. creaghi*, *C. brachyotis*, and *P. lucasi* were among the species contributing to the high positivity in the western region, where multiple CoV subgenera were detected, including *Sarbecovirus-rCoV*, *Nobecovirus-rCoV*, and *unclassified Borneo-α-CoV*. In Mulu, BtCoV detections were lower compared to the western region but still present across multiple species, particularly genus *Hipposideros* and *Rhinolophus*, which showed positivity for *Rhinovirus-rCoV* and *Unclassified Borneo-α-CoV*. In contrast, Niah exhibited only two positive cases, as reflected by the lighter shading in those sections of the heatmap. These detections were found in *C. brachyotis* and *M. magnater*, both testing positive for *unclassified Borneo-α-CoV* and *Minunacovirus-rCoV* respectively.

The results from the Q- and W- PCR assays are also displayed, highlighting the distribution of BtCoV detections across different molecular testing approaches. Q-assay yielded a higher number of positive detections, as indicated by more prominent dark-colored regions, while W assays detected fewer positive cases but still showed a complementary detection pattern. The overlap between Q & W assays confirms BtCoV positivity in certain bat species, with *C. brachyotis*, *P. lucasi*, and *R. creaghi* showing the strongest detection signals in both assays. This consistency between assays strengthens the reliability of CoV detection and suggests that these bat species may be important carriers of BtCoVs in Sarawak. A summary table (Table 4.7) has been added comparing the findings of this study with previous Malaysian BtCoV studies, in order to highlight the novel contributions of our work.

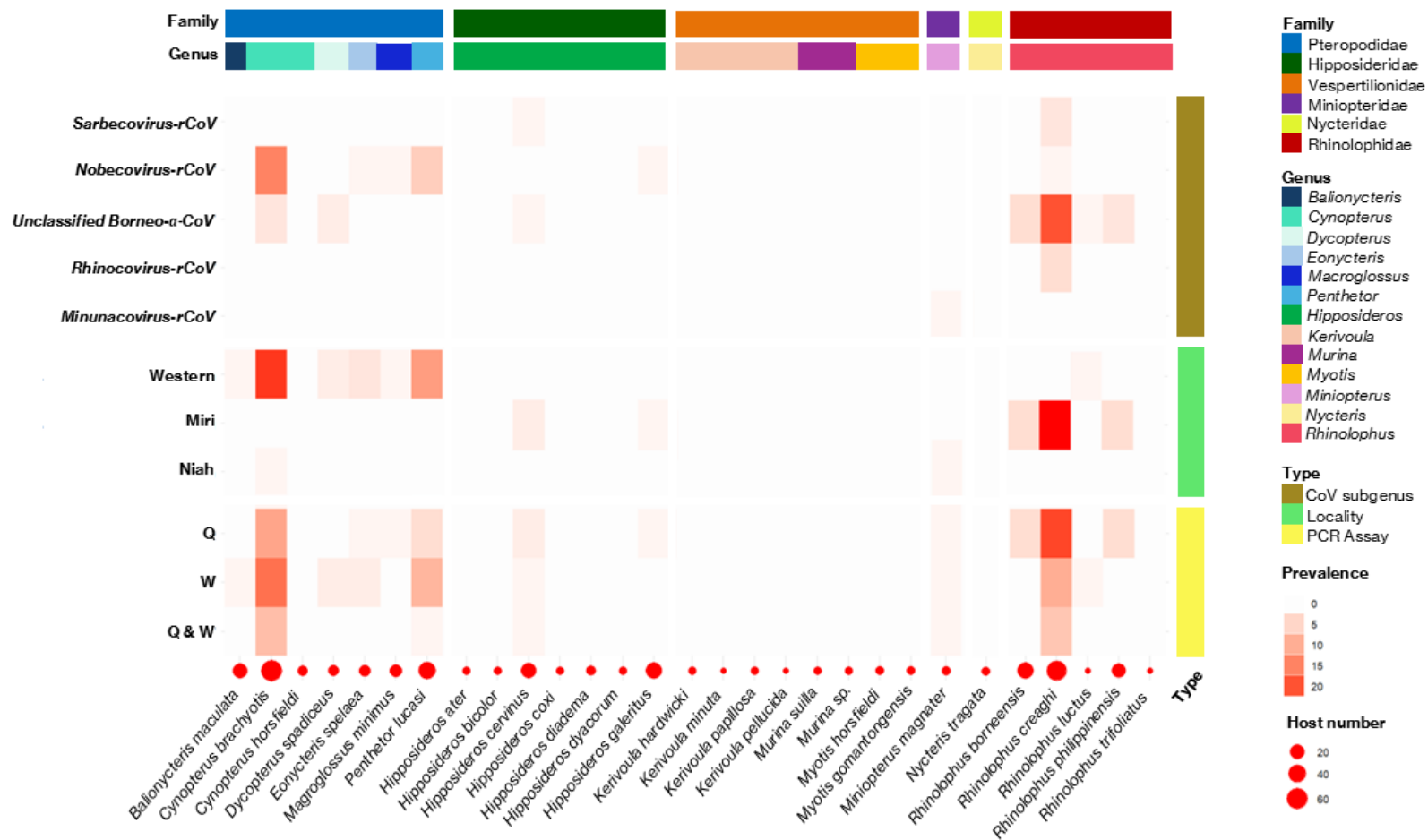


Figure 4.6: Positivity rates of bat CoVs across different bat species and localities using various assays in this study. Detection rates are represented by a color gradient from light to dark red, indicating low to high positivity. The orange circles at the bottom denote the number of hosts sampled, with circle sizes proportional to the sample count

Table 4.7: Summary of Key Studies on BtCoVs in Malaysia. The Table Includes Sample Types, Number of Samples Tested, CoV Prevalence, CoV Types Detected, and Highlights Each Study's Novelty

Study/Year	Location	Sample Type	Sample Size	CoV Prevalence	CoV Type	Novelty
Current study Habeebur-Rahman et al., (2025)	Sarawak (Western, Niah NP and Mulu NP)	Bat fecal, rectal swabs and urine	346	22.83% (n = 79/346)	α -CoV (<i>Minunacovirus-rCoV</i> , <i>Rhinocovirus-rCoV</i> , <i>unclassified Borneo-α-CoV</i>); β -CoV (<i>Sarbecovirus-rCoVs</i> , <i>Nobecovirus-rCoVs</i>)	First comprehensive study of BtCoV prevalence, diversity, and phylogeny in Sarawak, Malaysian Borneo, screening 346 fecal samples from 29 bat species across six families
Siew et al., (2023)	Krau Wildlife Reserve, Pahang	Bat oral and fecal	Not specified	Low (one positive sample)	β -CoV	Novel detection in insectivorous bats from a less sampled forest reserve in Peninsular Malaysia

Table 4.7 continued

Gilbert et al., 2023)	East Coast Malaysia (Kelantan, Terengganu and Pahang)	Bat oropharyngeal swabs	170	3%	β -CoV; SARS-CoV-2-like viruses	First evidence of SARS-CoV-2-like CoVs in bats from East Coast Malaysia
Habeebur-Rahman et al., (2023)	Sarawak (Wind Cave)	Dusky fruit bats (<i>Penthetor lucasi</i>) guano	39	38.5% (n = 15/39)	α -CoV; β -CoV (<i>Nobecovirus-rCoVs</i>)	First detection of novel CoVs in dusky fruit bats, highlighting underexplored frugivorous bats
Mahyudin et al., (2021)	Sabah	Bat rectal and throat	Not specified	Not specified	α -CoVs; β -CoVs	First molecular detection of diverse viral and bacterial pathogens in bats

Table 4.7 continued

Tan et al., (2021)	Sarawak (Wind Cave)	Bat guano	21	47.6% (n = 10/21)	α -CoV; β -CoVs	Discovery of novel CoV clades including zoonotic- related and unique Bornean lineages
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CHAPTER 5

DISCUSSION

5.1 The presence of BtCoV in Sarawak Chiropterans

The emergence of CoVs like SARS-CoV, MERS-CoV, SADS-CoV, and SARS-CoV-2 in the 21st century highlights the significant threat of zoonotic spillover from bats, often considered the primary reservoir (Chan-Yeung et al., 2003; Zaki et al., 2012; Zhou et al., 2020). Often, bats are pinpointed as the primary reservoirs for these viruses and blamed for outbreaks, as they are known to harbor closely related viral strains. Increasingly frequent discoveries of diverse α -CoVs and β -CoVs in bats necessitate better surveillance and preventative measures (Wong et al., 2019). While assays like the Quan and Watanabe tests are commonly used for CoV detection, their varying sensitivity and specificity pose challenges for accurate identification (Anis et al., 2021; Tan et al., 2023). This underscores the need for improved diagnostic tools and a deeper understanding of bat CoV diversity to mitigate future outbreaks.

A recent review analyzed 127 articles focusing on molecular studies of BtCoV. Of these, 85 articles (67%) were published between 2016 and 2022, highlighting the increasing research attention in recent years (Mulemena et al., 2025). The studies represented contributions from 54 countries, with China accounting for the largest share at 26% (33 out of 127 publications). These studies concluded that a combination of non-invasive and invasive sampling techniques, with non-invasive methods (nearly 75%) being the most used. The non-invasive sample types included guano, feces, urine, nasal secretions, throat swabs, and oral or saliva swabs, while invasive methods involved the collection of internal organs such as the liver, intestines, brain, lungs, spleen, kidneys, and heart. Many studies utilized

multiple sample types, with fecal samples being the most frequently collected, accounting for approximately 61.4% of the studies. Additionally, they documented the bat species that tested positive for CoVs, with a total of 324 species from 17 distinct families sampled in this study. Out of the 324 bat species examined, 206 species tested positive for CoVs. Among these, 78 species were from Asia, 64 species from Africa, 29 species from Europe, 25 species from North America, 13 species from South America, and 22 species from Australia. Similarly, in the present study, fecal samples were also used as the primary sample type, and CoVs were detected in multiple bat species across different families in Sarawak, Malaysia. This consistency with global trends underscores the reliability of fecal sampling for surveillance and highlights the important role of both frugivorous and insectivorous bats in maintaining CoV diversity in the region.

Focusing on western and northern Sarawak, our study explores the prevalence and diversity of CoVs in bats, confirming their importance as natural reservoirs for these viruses in a region with high bat diversity. Sarawak, Malaysian Borneo, harbors 80 known bat species spanning nine families (Pteropodidae, Emballonuridae, Megadermatidae, Nycteridae, Rhinolophidae, Hipposideridae, Vespertilionidae, Miniopteridae, and Molossidae), providing a rich ecological landscape for studying CoV (Abdullah et al., 2021). The variety of bat species and their CoVs aligns with research in other Southeast Asian countries, which has identified bats as key hosts for a broad spectrum of CoVs, including those capable of infecting humans, such as SARS-CoV and MERS-CoV (Anthony et al., 2017a; Hu et al., 2017). Filling a gap in the existing research, our study presents the first data from Sarawak using non-invasive bat samples, offering a distinct and potentially more comprehensive method for detecting CoVs in local bat populations compared to previous studies that mainly analyzed guano (Habeebur-Rahman et al., 2023; Tan et al., 2021) in

Sarawak, supporting the established understanding that bats are primary hosts to roughly 16 CoV subgenera (Latinne et al., 2020). Among the non-invasive samples collected, fecal samples yielded a higher detection rate, underscoring their value for surveillance.

The sampling strategies employed in this study were designed to maximize the detection of BtCoVs across different ecological regions in Sarawak. Bats were sampled from multiple locations, including the western region, Mulu NP, and Niah NP, representing diverse habitats such as cave systems, forest trails, and orchards (urban-fringed) areas. The combination of mist-netting and harp trapping allowed for the collection of a broad range of species, ensuring a representative dataset. This approach successfully captured frugivorous and insectivorous bats, contributing to the detection of BtCoVs across six bat families and 29 bat species. The species richness and Shannon diversity index provided insights into bat community composition across the three study sites. Niah NP exhibited the highest diversity, reflecting a more even distribution of species, whereas the western region was dominated by frugivorous bats. In contrast, Mulu NP showed higher representation of *Rhinolophus* and *Hipposideros* species, suggesting site-specific ecological factors influencing bat assemblages and, potentially, CoV maintenance.

The presence of BtCoV positivity in all three locations suggests that BtCoVs are widespread among Sarawak's bat populations, though the variation in detection rates may be influenced by differences in bat species composition and sample size. Although this study did not directly test the relationship between bat population density and CoV detection rates, regions in Sarawak where higher numbers of bats were sampled tended to yield more positive cases. A similar trend has been reported in other regions, such as China, where larger bat populations have been associated with greater viral detection (Cohen et al., 2023; Meyer et

al., 2024; Zhang et al., 2020). This suggests a link between bat population density and the probability of finding CoVs, implying that regions with more bats may be at higher risk for CoV emergence.

Several challenges were encountered during sample acquisition, which could have influenced the overall BtCoV detection rates. Weather conditions, particularly heavy rainfall, occasionally disrupted fieldwork and limited the number of bats captured per session. Sampling in Mulu NP was particularly challenging due to frequent heavy rainfall disrupting our efforts each day. However, leaving the traps overnight significantly improved our capture rate, ultimately making Mulu NP the site with the highest number of bats captured overall. The logistical difficulties of accessing remote sampling sites further constrained the ability to conduct repeated sampling in certain locations. Moreover, the availability of bats varied between sampling sites, with lower capture rates observed in Niah, which corresponded to the region's lower BtCoV detection rate. Another limitation was the difficulty in collecting sufficient fecal or rectal swab samples from certain species, potentially leading to an underestimation of viral prevalence in these bats.

5.2 Geographic Patterns of Sarawak Bats

Borneo is recognized for its rich biodiversity, boasting a diverse small mammal population of at least 186 documented species across several orders Chiroptera, Dermoptera, Pholidota, Rodentia, and Scandentia (Payne et al., 1985). However, Bornean forests face significant threats from habitat destruction, leading to declines in numerous bat species (Altringham, 2011). The bats are often forced to move closer to domestic areas due to habitat loss and lack of food, hence increasing the risk of virus transmission to other species including humans and farm animals (Smith & Wang, 2013). Despite existing research in Sarawak focusing on species distribution, taxonomy, and genetic data, the true abundance

and diversity of bats in the region remain underestimated (Khan et al., 2017; Mohd-Ridwan et al., 2018; Shazali et al., 2016). This gap in knowledge is particularly relevant when considering BtCoV prevalence, which varies across bat species and geographic locations, highlighting the importance of host specificity and regional differences in viral circulation. In this study, certain bat species exhibited higher positivity rates, suggesting their role as key reservoirs for BtCoVs. Notably, *R. creaghi* and *C. brachyotis* were among the species with frequent detections, particularly in western Sarawak and Mulu NP. The presence of multiple CoV subgenera in these species suggests that host ecology and behavior play a crucial role in viral maintenance and transmission. The roosting habits of these species, such as the tendency of many *Rhinolophus* to roost in caves and *Cynopterus* to roost in foliage or even human infrastructure, may contribute to differential exposure to CoVs.

In the western part of Sarawak, 142 bats were captured in this study with the majority belonging to the Pteropodidae family. The most common species was *C. brachyotis* (n = 53), a highly adaptable, fruit-eating bat frequently found in disturbed environments like agricultural areas, secondary forests, and urban green spaces (Rani et al., 2020). Its prevalence suggests that the surveyed locations in western Sarawak offer suitable conditions for foraging and roosting for this species. *C. brachyotis* is indeed a common bat species that can be found in various habitats, including oil palm plantations (Shafie et al., 2011). Some studies indicate that Perlis State Parks holds a relatively high diversity of small mammals, with *C. brachyotis* being the most dominant species (Fauzi et al., 2021). The prevalence of *C. brachyotis*, along with other frugivorous bats like *B. maculata* (n = 34) and *P. lucasi* (n = 17), suggests that the sampled locations offer a mix of open and forested environments capable of sustaining diverse bat populations. *B. maculata*, known as the spotted-winged fruit bat (Hodgkison & Kunz, 2006) typically inhabits primary and secondary forests, while

P. lucasi is often found in cave systems and forested areas. The abundance of frugivorous bats in this region emphasizes their ecological importance. Besides the fruit-eating bats, a small number of insectivorous bats from the Vespertilionidae family were also found: one *K. hardwickii* and one *K. papillosa*. *Kerivoula* bats, often called woolly bats typically inhabit forested areas, roosting among leaves or in tree holes (Mohd-Kanapiah et al., 2025; Sedlock et al., 2020). The low number of these bats captured suggests they might be less common in the surveyed areas, or that their secretive roosting and foraging habits make them harder to detect.

The bat community in the Niah NP region exhibited a high level of diversity, with 15 species representing six different families. Unlike other surveyed locations where a single species, such as *C. brachyotis*, dominated, Niah's bat population was more evenly distributed. This diversity can be attributed to the region's mix of forested and cave habitats, which provide a range of ecological niches that support both frugivorous and insectivorous bats. Notably, *R. creaghi* (n = 8) and *C. brachyotis* (n = 7) were the most frequently captured species, reflecting the suitability of these habitats for both cave-roosting and generalist bats, although capture frequency may also reflect the sampling effort applied at each site (see Materials and Methods).

The presence of multiple families, including Hipposideridae, Rhinolophidae, and Miniopteridae, highlights the importance of Niah's caves in sustaining species that exhibit strong roost fidelity. Bats such as *H. ater* and *R. philippinensis* are highly specialized in their roosting preferences, often relying on stable microclimates within caves (Mahyudin et al., 2022; Rajasegaran et al., 2018; Voss et al., 2016). In contrast, species like *C. brachyotis* and *B. maculata* are more adaptable, commonly found in both forests and disturbed habitats

(Basri et al., 2022; Mohd-Kanapiah et al., 2025; Pounsinsin et al., 2018; Shafie et al., 2011). This differentiation in habitat use suggests varying degrees of host specificity among bat species, where some are tightly linked to cave environments, while others can thrive in multiple landscapes.

A particularly notable finding was the capture of *M. gomantongensis*, a rare species with limited distribution records. Its presence in Niah suggests that these caves may serve as important refuges for less commonly encountered bat species. The coexistence of multiple bat families in the region also underscores the role of habitat complexity in shaping host diversity. Cave-roosting bats, such as *M. magnater* and *N. tragata*, may contribute to pathogen transmission dynamics due to their close colony structures and potential overlap with other species. In contrast, more mobile and generalist frugivores like *C. brachyotis* could act as bridging hosts, facilitating viral spillover between different environments (Han et al., 2015; Ruiz-Aravena et al., 2021; Sánchez et al., 2022). The high species richness observed in Niah further supports the idea that cave systems and surrounding forests play a crucial role in maintaining bat populations. Future studies focusing on host-specific pathogen associations could help clarify the role of different bat species in disease ecology, particularly in environments where diverse communities interact.

Mulu NP is distinguished by its remarkable bat diversity, with 154 individuals from 16 species captured during the survey. This high species richness, particularly the presence of unique and site-specific species, highlights Mulu's significance as a stronghold for bat biodiversity. The most frequently recorded species, *R. creaghi* (n = 55) and *H. galeritus* (n = 30), highlight the park's suitability for cave-dependent bat species, as both are known to prefer roosting in limestone cave systems. The discovery of six *Hipposideros* species,

including *H. bicolor*, *H. cervinus*, *H. coxi*, *H. diadema*, *H. dyacorum*, and *H. galeritus*, further emphasizes the importance of Mulu's cave network. Notably, *H. bicolor*, *H. coxi*, *H. dyacorum*, and *H. galeritus* were not found in any other surveyed locations, suggesting that Mulu provides essential microhabitats required for their survival. Similarly, the presence of *K. minuta* and *Murina* sp., both exclusively recorded in Mulu, indicates that the park supports species with specialized ecological requirements, particularly those that rely on undisturbed forested environments.

Mulu's exceptional bat diversity can be attributed to its extensive cave systems, formed through water drainage from the slopes of Gunung Mulu (Hall, 1996; Moulds & Anderson, 2014). These caves provide stable roosting conditions, including temperature and humidity levels that favour the persistence of multiple bat species. The complexity of Mulu's habitat likely contributes to niche partitioning, reducing interspecies competition and allowing a greater number of species to coexist (Azhar et al., 2013). Furthermore, the combination of primary rainforest and extensive cave networks in Mulu NP, as also observed in Merapoh caves (Peninsular Malaysia) and in Madai and Gomantong caves (Sabah), provides an ideal environment for both cave-roosting and foliage-roosting bat species, thereby enhancing overall species diversity (Fuad et al., 2024; Mohd-Kanapiah et al., 2025). Importantly, the presence of high bat densities within caves that are also accessible to tourists highlights a potential risk of zoonotic transmission (Ninwattana et al., 2025). Visitors may be exposed to bat excreta, aerosols, or contaminated surfaces within these enclosed environments, particularly where airflow is limited (Fornazari, 2021). While the ecological value of these caves is undeniable, such close human–bat interactions warrant careful management and biosafety considerations to mitigate potential health risks.

As part of the Heart of Borneo, a region known to support at least 100 mammal species, Mulu's unique assemblage of bats adds to the broader ecological significance of this conservation area (ASEAN Centre for Biodiversity, 2024). The high number of species recorded, along with the presence of site-restricted taxa, underscores the need for continued conservation efforts to preserve Mulu's pristine ecosystems (Azhar et al., 2013; Mc Arthur et al., 2024). Given the park's importance in sustaining a wide range of bat species, future studies should explore the ecological interactions between these bats and their environments, particularly in relation to their roles as pollinators, seed dispersers, and insect regulators. Additionally, assessing the presence of bat-borne viruses within this diverse community could provide valuable insights into host-pathogen dynamics in a highly biodiverse setting.

However, the present study is limited by its cross-sectional sampling design, which offers only a snapshot of viral diversity at a specific point in time. Viral shedding patterns in bats can vary seasonally, influenced by factors such as breeding cycles, food availability, and migration (Peel et al., 2019). As such, the findings reported here may not fully represent the temporal dynamics of CoV circulation within these bat populations. Longitudinal studies, incorporating repeated sampling across different seasons and years, are needed to capture the temporal variability of viral prevalence and better assess the risks of spillover (Hayman et al., 2012). Similarly, geographic variation in habitat type, bat species composition, and human-wildlife interfaces could contribute to differences in CoV prevalence and diversity across sites (Anthony et al., 2017; Latinne et al., 2020). Future studies incorporating temporal and spatial dimensions would therefore provide a more comprehensive understanding of the ecological drivers of BtCoV circulation.

5.3 Insights from the Phylogenetic Analysis

The phylogenetic analysis in this study represents the first dataset in Sarawak providing a detailed classification of different BtCoV clades, offering new insights into their diversity and evolutionary relationships. A total of 79 BtCoVs were successfully detected, while 13 samples were unsuccessful in amplification or sequencing. These BtCoVs were grouped into six distinct CoV subgenera, which are two *unclassified Borneo-α-CoVs* and unclassified CoVs within the subgenera *Minunacovirus*, *Rhinolophus*, *Nobecovirus*, and *Sarbecovirus*. This comprehensive analysis not only refines our understanding of BtCoV diversity in Malaysian bat populations but also highlights the evolutionary dynamics of these viruses. By comparing our sequences with global BtCoV datasets, we provide critical insights into host-virus associations, potential cross-species transmission, and the phylogenetic placement of Malaysian BtCoVs within the broader CoV evolutionary framework. These findings contribute to a more robust foundation for future surveillance and zoonotic risk assessments in the region.

A significant discovery from this study is the detection of a *Minunacovirus-rCoVs* (*Clade A1*) in *M. magnater* bats captured in Niah NP. Genetic analysis showed that the identified strain clustered closely with *Miniopterus* strains from China and Hong Kong, sharing 91.99 to 93.20% similarity (Chu et al., 2006, 2008; Han et al., 2023). This aligns with previous research indicating that the *Minunacovirus* subgenus is primarily associated with the *Miniopterus* genus (Han et al., 2023; Li et al., 2024). The identification of a potential precursor virus to SARS-CoV in *M. magnater* is particularly concerning, highlighting the potential for new zoonotic CoVs to emerge (Dobson, 2005). Given the diverse habitats of this bat species, further research on the *Miniopterus* genus in Sarawak is crucial to evaluate

their potential risks in zoonotic transmission, especially considering their known association with various recombinant strains.

Analysis of α -CoV clades in both Q and W assays revealed interesting patterns among rhinolophids (horseshoe bats). *Rhinolophus* bats are known reservoirs for CoVs (Fan et al., 2019; Latinne et al., 2020). In the Q-assay, CoVs found in *R. borneensis*, *R. creaghi*, and *R. philippinensis* clustered within the *unclassified Borneo- α CoVs (Clade A2)*. This is notable because *Rhinolophus* species are recognized reservoirs for SARS-related CoVs. This clade shares genetic similarities with sequences from Sabah, Malaysia Borneo, suggesting potential ecological overlap or behavioral similarities between these bat populations. Communal roosting and feeding habits in these bats may facilitate viral circulation. Stable *Rhinolophus* populations in dense forest habitats may also support viral persistence (Senawi & Kingston, 2019). The observed genetic similarity suggests limited CoV divergence within these bat populations. In contrast, the W-assay identified a distinct clade, *the unclassified Borneo- α CoVs (Clade A3)*, linking CoVs from both frugivorous and insectivorous bats, including previously identified BorneoAlpha-1 and BorneoAlpha-2 (Habeebur-Rahman et al., 2023; Tan et al., 2021). This indicates shared viral reservoirs among bat populations across western Sarawak, possibly due to frequent interspecies interactions in habitats with limestone caves and dense vegetation. Such connectivity may promote CoV persistence and circulation.

Another notable clade identified was the *Rhinocovirus-rCoVs (Clade A4)* in the W-assay. Here, *R. creaghi* clustered closely with *Rhinocovirus* strains like BtCoV/HKU2 and SADS-rCoV, previously found in *R. affinis* and *R. pusillus* in China. The subgenus *Rhinocovirus* appears to be host-specific within rhinolophid bats (Latinne et al., 2020). The

HKU2 bat CoV is significant due to its close relation to viruses with zoonotic potential, such as SADS-CoV in pigs. Its discovery in *Rhinolophus* bats highlights the ability of bat viruses to jump between species and potentially affect agricultural animals and even humans (Zhou et al., 2018; Gong et al., 2017; Pan et al., 2017). The co-detection of α -CoVs and β -CoVs in *R. creaghi* marks this species as an important reservoir of diverse CoVs, emphasizing the need for continued monitoring in these regions.

The study detected CoVs in several frugivorous bat species, including *C. brachyotis*, *D. spadiceus*, *E. spelaea*, *M. minimus*, and *P. lucasi*. The findings indicated a higher positivity rate for CoVs in frugivorous bats, specifically *C. brachyotis* and *P. lucasi*, compared to insectivorous bats when using the W-assay. This result aligns with Watanabe (2010), where four out of six tested bat species in their study were frugivorous. This higher detection rate may be because the primer is more effective for frugivorous bats or because a greater sampling effort was concentrated on habitats where frugivorous bats are more prevalent. If we account for these potential biases, the increased detection rate might be due to dietary exposure from virus-laden fruits or social behaviors that promote viral transmission. Another study suggests that the effectiveness of certain primers can decrease if there are mismatches in the primer sequences (Holbrook et al., 2021).

Interestingly, The CoVs found in the Pteropodidae family (frugivorous bats) display a variety of α -CoVs and β -CoVs. In the W-assay, fruit bat CoVs clustered with insectivorous bats within the *unclassified Borneo- α -CoVs* subgenus (*Clade A3*). For both the Q- and W-assays, most CoVs from frugivorous bats grouped into the *Nobecovirus-rCoVs* (*Clade B1*), a subgenus known for its host specificity to the Pteropodidae family. In our previous study (Habeebur-Rahman et al., 2023), we described the BorneoBeta-3 CoV clustered with

Rousettus bat GCCDC1 related CoVs (RoGCCDC1r-CoVs) and *Pteropus* sp. from Southeast Asia and India. It was reported that *Rousettus* bat CoV GCCDC1 (RoBat-CoV GCCDC1) initially found in *Rousettus leschenaultii* in Yunnan province in 2016, was later identified in *E.spelaea* populations that co-roost with *R. leschenaultii* (Luo et al., 2018). Of one interest, the CoV found in *E. spelaea* clustered with the BtCoV/HKU9 strain (EF065516) from China. Although *R. leschenaultii* has not been documented in Sarawak, it is present in Peninsular Malaysia (Abdullah et al., 2021). While the zoonotic potential of the subgenus *Nobecovirus* remains unclear, viruses like RoBat-CoV GCCDC1 and HKU9 carry uncertain risks. Previous research has reported recombination is common in this subgenus, especially in the GCCDC1 lineage in Asia, which has a *p10* gene insertion from an orthoreovirus (Huang et al., 2016). This recombination potential increases the risk of new viral strains emerging within host populations, raising the likelihood of spillover events and complicating efforts to control emerging CoV outbreaks.

Within the *Sarbecovirus-rCoVs* (Clade B2) observed in the W-assay, this study identified two β -CoV clustering patterns. First, the previously described BorneoBeta-1 sequence clustered with *Hipposideros* spp. CoVs from Mulu NP and Sabah (BtCoV/PREDICT CoV-51/PSW00163). The pooled guano samples from Wind Cave's roosting site indicate a complex co-roosting dynamic among *Hipposideros* bat species, which may explain the shared CoV sequences detected in these samples (Tan et al., 2021). Second, bat CoV from *R. creaghi* clustered with BtCoV/PREDICTCoV-84/PSW01983, formed a sister clade to the *Sarbecovirus* subgenus. This observation underscores the importance of pursuing full-genome sequencing for these sequences to better understand their genomic characteristics and facilitate accurate classification. A particularly significant finding from our study is the absence of *Sarbecovirus*-like CoVs in the sampled bat

populations, suggesting no current evidence of reverse zoonotic transmission from human CoVs to bats in Sarawak. This supports the hypothesis that the detected non-human CoVs likely originated from endemic bat species, notably within the Rhinolophidae family represented in this analysis.

These phylogenetic insights highlight not only the evolutionary diversity of BtCoVs in Sarawak bats but also their potential relevance beyond ecological boundaries. While our analyses focus on viral diversity and host associations, the implications extend to the interface of wildlife, human activities, and public health. This provides a strong rationale to frame the findings within a One Health perspective, which emphasizes the interconnectedness of ecological processes, zoonotic risks, and health security.

5.4 Integrating a One Health Perspective

The detection of BtCoVs in Sarawak chiropterans highlights their importance as reservoirs of viral diversity, with implications that extend beyond ecology. From a One Health perspective, such findings are relevant not only for understanding viral diversity but also for assessing how ecological and human factors may influence their circulation (Anthony et al., 2017; Plowright et al., 2017). Geographic differences observed among bat assemblages, particularly in areas where natural habitats interface with agricultural or peri-urban landscapes, suggest that environmental change may affect opportunities for virus maintenance and transmission. Human activities such as land-use modification and cave visitation may alter bat community structure and behavior, thereby influencing viral dynamics (Hayman, 2016). These patterns indicate that geographic context is an important factor when interpreting the presence and diversity of BtCoVs.

Phylogenetic analyses further demonstrated that Sarawak bats harbor both previously recognized and novel CoV lineages, including sequences clustering within the *Sarbecovirus* group. Given the historical significance of *Sarbecoviruses*, these findings reinforce the importance of continued genomic surveillance to track evolutionary patterns. Linking such phylogenetic insights with ecological data and land-use information would provide a more comprehensive basis for evaluating the potential implications of viral diversity (Destoumieux-Garzón et al., 2018). Overall, this study demonstrates the value of applying a One Health framework, whereby viral discovery is integrated with ecological understanding and public health preparedness. Strengthening interdisciplinary collaboration by connecting ecological field surveys, virological screening, and health system monitoring will enhance the capacity to interpret CoV diversity in wildlife and its relevance to broader health and conservation agendas.

5.5 Comparison of Q-Assay and W-Assay in BtCoV Detection

A variety of assays have been used in the detection of many different viruses, including PCR, serological tests and virus isolation (Calisher et al., 2006; Canuti et al., 2011; Diakoudi et al., 2019; Han et al., 2017; Kohl & Kurth, 2014; Kuno, 2001; Male et al., 2016; Megali et al., 2010; Monath, 1999; Reynes et al., 2005; Sendow et al., 2006). These methods have been instrumental in identifying viruses across a wide range of hosts, including insects, plants, fungi and bacteria, reflecting the diverse dietary habits of bats (Donaldson et al., 2010; Ge et al., 2012; Li et al., 2010). In this study, two molecular assays, the Q-assay and W-assay, were employed to detect BtCoVs in frugivorous and insectivorous bats. These protocols are frequently used as broad-range PCR assays to detect CoV. Despite their widespread use in various ecological studies, these assays have not been thoroughly investigated in Sarawak. This is concerning because Southeast Asia, including Sarawak, is

a high-risk zone for emerging infectious diseases due to its extensive natural habitats and ongoing ecosystem fragmentation. By deploying both assays, this research not only provides a comparative analysis of their performance but also contributes to valuable insights into the prevalence and diversity of BtCoVs in Sarawak, helping to refine molecular diagnostic techniques for future studies.

The comparison of the Q-assay and W-assay in detecting BtCoVs highlights both the strengths and limitations of these molecular techniques. The fair agreement observed between the two assays, indicated by a Kappa value of 0.286, suggests that while they can detect BtCoVs, their classifications are not entirely consistent. The fact that only 18 samples were positive in both assays (W+Q+) while 270 samples were negative (W-Q-) implies that some samples may be preferentially detected by one assay over the other. This variation in detection could be due to differences in primer specificity, amplification efficiency, or the genetic diversity of the targeted CoV sequences. As BtCoVs are highly diverse, some strains may align more closely with the primers used in one assay while remaining undetected by the other.

Despite the observed differences in detection rates, the McNemar test results indicate that there is no statistically significant difference between the performance of the Q-assay and W-assay ($p = 0.512$). This suggests that both assays are comparably effective in detecting BtCoVs, albeit with some discrepancies. The lack of a significant difference implies that neither assay is clearly superior, and their combined use could enhance overall detection sensitivity. If one assay is more sensitive to a specific CoV lineage, using both in parallel could provide a more comprehensive picture of viral diversity. Additionally, the fair

agreement between assays highlights the complexity of BtCoV detection and the need for refined molecular tools that can more reliably identify a broad range of CoVs.

In this study, we observed a discordant result for the sequence MNP063, where the Q-assay assay and W-assay classified the sequence differently. The Q-assay grouped MNP063 within the *unclassified Borneo- α -CoVs* (*Clade A2*), showing a 92.51% sequence similarity to another α -CoV from Malaysia. However, the W-assay classified MNP063 in the *Sarbecovirus-rCoVs* subgenus (*Clade B2*) with a higher sequence similarity of 97.83%. This discrepancy could arise from the assays targeting different viral genetic regions, with the Q-assay potentially more sensitive to certain α -CoVs and the W-assay better at detecting β -CoVs, especially *Sarbecoviruses* (like RaTG13 and BANAL-20-52), to which MNP063 is closely related. Interestingly, two other sequences from the same species (*R. creaghi*) were consistently grouped by both assays, although in slightly different subgroups (*unclassified alpha-CoVs* in Q-assay and *Rhinocovirus-rCoVs* in W-assay). This suggests assay biases in categorizing certain viruses, even with agreement on other sequences. The discordance between the assays for MNP063 highlights the genetic diversity of bat CoVs, especially in regions like Borneo, where viruses may not fit neatly into existing categories.

Given the observed variations between the assays, future studies could explore modifications to improve assay sensitivity and specificity. Expanding the genetic regions targeted by the assays or designing primers with greater coverage across different CoV lineages may help minimize discrepancies. Additionally, validation of these assays with full-genome sequencing could provide deeper insights into potential biases in detection. One limitation of the present study is that the analysis was based on partial RdRp sequences, which restricts the ability to fully resolve the evolutionary relationships and potential

recombination events among BtCoV. While this approach provides a reliable preliminary assessment of viral diversity, it does not capture the full genomic context required to infer functional implications or detect genome-wide variations (Latinne et al., 2020; Woo et al., 2012b). Future studies should therefore incorporate whole-genome sequencing (WGS) to provide a more comprehensive understanding of viral diversity, evolutionary dynamics, and zoonotic potential. The use of WGS would not only improve phylogenetic resolution but also enable the detection of recombination hotspots and adaptive mutations that are critical for assessing spillover risk (Anthony et al., 2017).

Moreover, WGS would facilitate comparisons across different CoV genes, such as the S protein, which plays a key role in receptor binding and host range determination (Letko et al., 2020). This is particularly important as partial RdRp sequences, while conserved, may underestimate functional diversity and adaptation potential (Hu et al., 2017). WGS also allows for the identification of accessory genes and structural variations that may influence pathogenicity, transmissibility, and host adaptation. Importantly, having complete genomes would enable integration into global databases, such as GISAID or GenBank, thus contributing to broader comparative analyses and surveillance efforts (Elbe & Buckland-Merrett, 2017). In addition, WGS data could support molecular clock analyses to estimate divergence times and track the evolutionary history of CoVs within local bat populations. This temporal dimension is crucial for understanding whether observed lineages represent long-standing endemic strains or more recent introductions (Boni et al., 2020). By linking genomic data with ecological and epidemiological information, future research can better identify high-risk viral strains and inform targeted monitoring strategies (Plowright et al., 2017).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study provides the first comprehensive investigation into the prevalence, diversity, and phylogenetic relationships of BtCoVs in Sarawak, Malaysian Borneo. By screening 346 fecal samples from 29 bat species across six families, we identified a diverse range of BtCoVs, classified within six distinct clades of α -CoVs and β -CoVs. Among these, two unclassified Borneo- α -CoVs and four classified CoVs within the subgenera *Minunacovirus*, *Rhinacovirus*, *Nobecovirus*, and *Sarbecovirus* were detected. The comparative evaluation of the Q-assay and W-assay provided critical insights into the efficiency of molecular detection methods for BtCoVs. Although both assays effectively detected CoVs, they exhibited fair agreement ($\kappa=0.286$, $p<0.001$) with no statistically significant difference in detection performance ($p>0.05$). The observed variation in sensitivity and specificity highlights the importance of employing multiple assays to improve CoV detection and classification. Additionally, the use of fecal samples as a non-invasive method proved to be a valuable approach for BtCoV surveillance, demonstrating its potential for future ecological and epidemiological studies. To further refine these findings, WGS of the unclassified CoV clades is recommended, as it would improve taxonomic resolution and clarify their evolutionary placement. Expanding WGS efforts would also enable more robust comparisons with global CoV datasets, contributing to a deeper understanding of CoVs diversity in bats.

Given the high biodiversity of bats in Sarawak and the increasing risks of zoonotic spillover due to habitat fragmentation and human-wildlife interactions, continued

surveillance of BtCoVs is essential. This study contributes valuable baseline data on CoV diversity in Sarawak, reinforcing the need for ongoing research and improved public health preparedness to mitigate potential outbreaks. Our findings highlight the significant viral diversity within specific bat species, particularly *Miniopterus magnater* and *Rhinolophus creaghi*, which harbor CoV strains closely related to known zoonotic viruses such as SARS-CoV. The observed differences in detection rates between the Q-assay and W-assay suggest that relying on a single assay may not be sufficient for comprehensive BtCoV surveillance. The combination of both assays enhances the ability to capture a broader spectrum of CoVs, including potentially novel strains. This underscores the importance of refining diagnostic methods to improve CoV detection accuracy and reliability. Furthermore, this study identifies key reservoir species and geographic hotspots for BtCoVs in Sarawak. Species such as *R. creaghi* and *C. brachyotis* were found to have high BtCoV positivity rates, particularly in Mulu NP and western Sarawak. These areas provide ecological conditions conducive to virus circulation, likely driven by high bat species diversity and environmental factors that support host-virus interactions. The high frequency of BtCoV detections in western Sarawak and Mulu NP suggests that these regions may act as key viral reservoirs, emphasizing the need for targeted surveillance and monitoring.

In addition, ecological modeling represents a powerful approach to predict the spatial distribution of BtCoVs and identify areas where virus maintenance is most likely to occur. By integrating data on bat species distribution, habitat types, and patterns of land-use change, predictive models could help delineate regions where environmental conditions favor host-virus interactions. Such models would also allow scenario testing, for example, assessing how continued deforestation or agricultural expansion may alter bat distribution and viral dynamics. Incorporating these approaches into BtCoV research could strengthen both

conservation planning and public health preparedness by highlighting ecological contexts in which viral diversity and transmission potential are concentrated.

The high prevalence of BtCoVs in specific species and locations carries important implications for disease ecology and potential spillover risks. Land-use changes, such as deforestation and urban expansion, may alter bat population dynamics and shift viral reservoirs closer to human settlements. For instance, habitat fragmentation in western Sarawak may drive synanthropic bat species like *C. brachyotis* to roost in urban and agricultural areas, increasing the likelihood of human-bat interactions and potential virus transmission. The popularity of cave tourism in Mulu and Niah NP may inadvertently expose visitors to environments with high viral loads, highlighting the relevance of our habitat-based analysis. Although direct transmission of CoVs from bats to humans is uncommon, zoonotic transmission often occurs through intermediate hosts. Examining the habitat-level distribution of CoVs therefore complements existing arguments in this study by clarifying the ecological settings in which viral maintenance and potential transmission pathways are most likely to develop. Such information is also important for conservation and management, as different habitats (e.g., caves, orchards, and forest edges) not only shape bat community composition and viral diversity but also determine the frequency and intensity of human–bat interactions. Integrating ecological surveillance with an understanding of land-use change and tourism pressure can provide a more comprehensive framework for assessing future risks and prioritising both public health and biodiversity conservation. Longitudinal studies would provide valuable insights into the temporal dynamics of BtCoV circulation in bat populations. Seasonal fluctuations in resource availability, breeding cycles, and migratory behavior are known to influence bat population structure and contact rates, which in turn can affect viral transmission and maintenance. By sampling across different

seasons, future studies could determine whether BtCoV prevalence varies in response to ecological drivers such as rainfall, fruiting periods, or roosting density.

In conclusion, this study provides critical insights into BtCoV diversity, host associations, and geographic distribution in Sarawak, contributing to the broader understanding of CoV ecology in Southeast Asia. The findings emphasize the need for continuous monitoring of bat populations, refinement of molecular detection methods, and a One Health approach to mitigate future zoonotic risks.

6.2 Recommendations

Recommendations to enhance and standardize capture rates of bats can be considered in future studies. Some species demonstrated trap avoidance, which limited sample size; therefore, integrating multiple trapping methods (e.g., harp traps, mist nets, acoustic monitoring) across different habitats could improve representativeness. Standardizing these approaches would reduce sampling bias and strengthen the reliability of ecological and virological conclusions. To ensure the reliability of the data, strict quality control measures were implemented throughout sample collection and processing. Field equipment, including mist nets, gloves, and sample collection tools, was thoroughly disinfected with 70% ethanol between captures to prevent cross-contamination between individuals. Samples were immediately stored in VTM to stabilize nucleic acids and prevent degradation. This step was crucial, as RNA viruses such as CoVs are highly susceptible to degradation if not properly preserved. The samples were then stored and transported at low temperatures until further processing in the laboratory. This cold-chain storage ensured that viral RNA remained intact, allowing for accurate detection through RT-PCR assays. In the laboratory, negative controls and multiple PCR replicates were used to minimize the risk of false-positive results.

Additionally, extraction blanks and no-template controls were included during RNA extraction and PCR amplification to detect potential contamination. These measures ensured that the BtCoV detections reported in this study were not the result of laboratory errors but reflected the actual presence of CoVs in the sampled bat populations.

Moreover, the fair agreement observed between the Q-assay and W-assay suggests the need for refined molecular tools with improved sensitivity and specificity. Our study suggests that the Quan and Watanabe primers are not true pan-CoV primers. Consequently, previous prevalence studies (Anthony et al., 2017b; Kumakamba et al., 2021; Lacroix et al., 2017; Latinne et al., 2020; Quan et al., 2010; Wacharapluesadee et al., 2015, 2024; Watanabe et al., 2010) using these primers may have underestimated the actual prevalence of CoVs. To address this limitation, alternative amplicon sequencing strategies need to be developed to support LMIC researchers in their efforts to identify novel pathogens. For example, in human papillomavirus (HPV) detection, the degenerate MY09/11 primer set was found to have lower sensitivity for detecting HPV in primary specimens (Qu et al., 1997). An improved version, PGMY09/11, which uses a cocktail of specific primers, demonstrated significantly higher sensitivity (Gravitt et al., 2000). Similarly, pan-CoV or family-wide PCR approaches may benefit from a multiplexed cocktail of specific primers targeting the same binding sites rather than relying solely on a single pair of highly degenerate primers. Beyond targeted PCR, WGS offers great promise for identifying novel pathogens and classifying unassigned CoV clades. Yet, its high cost remains a barrier in many LMICs. Development of more affordable amplicon sequencing strategies could therefore enhance capacity in LMICs, while metagenomic analyses would provide broader insights into viral diversity beyond CoVs.

One important step would be to integrate BtCoV monitoring into national wildlife health programs, ensuring that virus surveillance is conducted systematically alongside biodiversity and conservation assessments. Embedding such monitoring into existing frameworks would enhance early detection capacity and provide long-term datasets essential for understanding viral trends in wildlife populations. In parallel, closer collaboration with public health agencies is needed to translate ecological findings into risk assessments that are relevant for human health. For instance, high-tourism cave areas such as Mulu and Niah NP attract large numbers of visitors who may come into close proximity with bat populations. Joint initiatives between conservation authorities and health agencies could establish evidence-based guidelines for tourism management, including visitor education, cave access protocols, and monitoring of bat colonies in heavily visited sites. Such cross-sectoral coordination would help balance biodiversity conservation, tourism development, and public health preparedness.

Future research should focus on long-term surveillance, habitat changes, and viral evolution to further elucidate the mechanisms driving CoV emergence and potential spillover events. While long-term surveillance is ideal for monitoring viral dynamics, in practice it is often constrained by logistical, financial, and manpower limitations. Nonetheless, periodic or targeted surveillance, particularly in ecologically sensitive habitats and regions of high bat diversity, can still provide valuable insights into habitat changes, viral evolution, and the ecological mechanisms that underlie CoV circulation.

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APPENDICES

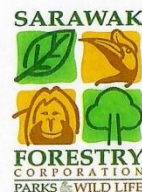
Appendix A: Journal Publications

1. **Habeebur-Rahman, S.P.**, Khan, F.A.A., Mohd-Azlan, J., Melvin, G. & Tan, C.S. (2025). Comparative Analysis of Quan and Watanabe Pan-Coronavirus Assays for Bat Coronavirus Diversity in Sarawak, East Malaysia. *Journal of Medical Virology*, 97(5), e70389.
2. Tahar, A.S., **Habeebur-Rahman, S.P.**, Yaman, K., Ngui, R., Lim, A.L.L, Edwin, J., Saboden, N.S., Manggi, T. & Tan, C.S. (2025). Genetic diversity of *Ancylostoma ceylanicum* and first molecular detection of *Ancylostoma braziliense* in stray dogs from Sarawak, Malaysia. *Scientific Reports*, 15, 14586.
3. Tan, C.S., Adrus, M., **Habeebur-Rahman, S.P.**, Azman, H.I.M., & Abang, R.A.A. (2024). Seroevidence of SARS-CoV-2 spillback to rodents in Sarawak, Malaysia Borneo. *BMC Veterinary Research* 20(1), 161.
4. Arios, Y.P., Pamungkas, J., Wibawan, I.W.T., Iskandriati, D., Tan, C.S., & **Habeebur-Rahman, S.P.** (2024). Presence of neutralizing antibodies to SARS-CoV-2 in cats and dogs transported to Indonesia. *Advances in Animal and Veterinary Sciences*.
5. **Habeebur-Rahman, S. P.**, Noni, V., Khan, F. A. A., & Tan, C. S. (2023). Detection of novel coronaviruses from dusky fruit bat (*Penthetor lucasi*) in Sarawak, Malaysian Borneo. *Veterinary Medicine and Science*, 9(6), 2634-2641.
6. Tan, C.S., Bandak, D.B., **Habeebur-Rahman, S.P.**, Tan, L.T., & Lim. L.L.A. (2023). Serosurveillance of SARS-CoV-2 in companion animals in Sarawak, Malaysia. *Virology Journal* 20(1), 176.

Appendix B: Sarawak Forestry Corporation (SFC) Permit Approval

(page 1/5)

Your Ref. : 2022004
Our Ref. : SFC.810-4/6/1 (2022) - 068
Date : 13 July 2022



Assoc. Prof. Dr. Tan Cheng Siang
Faculty of Medicine and Health Sciences
Universiti Malaysia Sarawak
Jalan Dato Mohd Musa
94300 Kota Samarahan, Sarawak

Dear Sir/Madam,

**PERMISSION TO CONDUCT RESEARCH ON BIOLOGICAL RESOURCES – PERMIT
SFC.810-4/6/1 – 067 AND PARK PERMIT NO. WL 23/2022**

Greetings from SFC!

The application for the above subject matter refers.

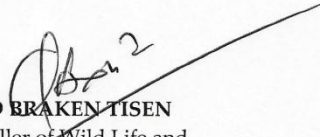
Enclosed herewith is the permission and approval for you and your team member to conduct research in Sarawak. This approval letter is to be returned to the Controller upon expiry of its validity period.

To conduct research outside Totally Protected Areas (TPAs), you and your team member shall obtain permission from manager or authority in charge of the area before commencement of research activity.

Members of your research group are also required to liaise and report to Sarawak Forestry Corporation (SFC) upon collection of biological resources samples/specimens to ensure that the Wild Life Protection Ordinance, 1998 are complied with accordingly.

Thank you.

I CARE


OSWALD BRAKEN TISEN
for Controller of Wild Life and
National Parks & Nature Reserves
SARAWAK

nr/

“NEW FRONTIER IN BIODIVERSITY CONSERVATION”

SARAWAK FORESTRY Corporation
Lot 218, KCLD, Jalan Tapang, Kota Sentosa, 93250 Kuching
Tel: (+6) 082 610088 Fax: (+6) 082 629744 www.sarawakforestry.com

ID: 2022004

Your Ref. : 2022004

Our Ref. : SFC.810-4/6/1(2022) – 067

Date : 13 July 2022



Assoc. Prof. Dr. Tan Cheng Siang
Mdm. Sultana Parvin Binti Habeebur Rahman
Assoc. Prof. Dr. Mohd Azlan Jayasilan Bin Abdul Gulam Azad
Assoc. Prof. Dr. Faisal Ali Bin Anwarali Khan
Mr. Azroie Denel
Faculty of Medicine and Health Sciences
Universiti Malaysia Sarawak
Jalan Dato Mohd Musa
94300 Kota Samarahan, Sarawak

Dear Sir/Madam,

PERMISSION TO CONDUCT RESEARCH ON BIOLOGICAL RESOURCES/COLLECT SAMPLES OF BIOLOGICAL RESOURCES FOR RESEARCH:- INVESTIGATING THE PHYLOGENETIC DIVERSITY OF SARS-CoV LIKE VIRUSES IN VARIOUS SPECIES OF BATS IN SARAWAK

Greetings from SFC!

The above subject matter refers.

2. With regards to your application for the above subject, I am pleased to inform that permission is hereby granted for you and your team members to conduct research and collect samples of biological resources within the area as per table below:

Species		Location	Quantity
Scientific Name	Type of Organism		
	Bat	National Parks & Nature Reserves: - Tanjung Datu NP - Samungam WS - Bungo Range NP - Gunung Gading NP - Gunung Pueh NP - Kubah NP - Matang WC	5 specimens per species (rectal, throat swabs, faeces, urine, wing puncture and blood sample)

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ID: 2022004

		• Bako NP • Gunung Gading NP • Sama Jaya Nature Reserve • Santubong NP • Derek Krian NP • Sabal FR • Bukit Lima Nature Reserve • Maludam NP • Batang Ai NP • Kuching Wetland NP • Talang Satang NP • Pulau Tukung Ara & Banun WS • Similajau NP • Lambir Hills NP • Niah NP • Mulu NP • Usun Apau NP Human-animal interface area (attics/roofs, caves, orchards) • Batang Lupa area • Mount Penrissen • Borneo Highlands • Kota Samarahan • Bau Limestone • Penrissen-Padawan-Bau • Engkelili • Kapit • Long Lellang • Bario • Bakelalan • Murud	Note: (a) Duplicate of specimens to be deposited at (1) Sarawak Forestry Corporation, (2) Sarawak Biodiversity Centre, (3) Institute of Medical Research, and (4) UNIMAS (b) To inform SFC sampling/field trip schedule.
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3. This permission is **valid for one (1) year**, effective from **13 July 2022 – 12 July 2023**. This permit shall be surrendered to the Controller of Wild Life, Sarawak or any officer duly authorized by him upon the expiry thereof.

4. The permission is granted on a 'personal-to-holder' basis and shall not be assigned. The holder must produce this letter/permit for inspection when called upon by any member of the issuing authority or any other enforcement agencies.

5. This is not a permit for the holder to conduct research on medicinal, pharmaceutical, therapeutic, nutritional or agricultural properties of the biological resources collected.

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6. The permission may be summarily withdrawn in the event of non-compliance with the condition set therein.
7. The authority is at liberty to assign one or more local counterpart to accompany the holder and to participate in research and in the collection of the biological resources.
8. The issuing authority and/or the State Government of Sarawak shall not be liable for any injuries or loss of life caused or arising from activities related to the implementation of the research and the collection of the biological specimens.
9. The holder shall bear all the expenses incurred on the research conducted unless otherwise stated.
10. The holder shall notify the issuing authority of any previously undescribed species found and the proposed name.
11. The holder is to deposit a duplicate copy/a voucher of the biological resources collected with Sarawak Forestry Corporation, Kuching, Sarawak.
12. The holder shall brief and debrief member of the issuing authority in charge of the area, prior to and before leaving the research areas site, and a final briefing on the research finding shall be given to the authority upon completion of the research activities.
13. The holder shall submit three (3) copies of brief summary of research finding and a preliminary report including data and images either in English or Bahasa Malaysia upon expiry of the permit to the issuing authority.
14. The holder shall comply with all the written laws, rules and regulations made thereunder pertaining to removal of the biological resources from the state of Sarawak.
15. The materials collected should neither be commercialized nor transferred to a third party without prior informed consent or prior consent in writing of the Sarawak Government.
16. Export of specimens requires an export permit from the Controller of Wild Life, Sarawak, with a fee as stated in the Wild Life Protection Rules, 1998 and a copy of the research permit to be attached to the application. Export of plant and animal specimen(s) are only allowed in it dried form. (if any)
17. Collection of samples from plank walks and trails around tourism zone are not allowed. Collection of flora and fauna samples inside Totally Protected Areas (TPAs) shall be guided by staff of Sarawak Forestry Corporation.
18. Specimens taken out from the park must be verified by Park Warden or Officer-in- Charged.

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ID: 2022004

19. The holder, who intend to collect a totally protected animal MUST acquire written permission or consent from the Controller of Wild Life for totally protected animal specified in Para 2.

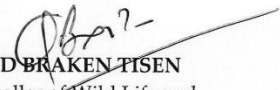
20. The holder who intends to conduct research in plantation area or outside Totally Protected Areas (TPAs) shall obtain permission from manager or authority in charge of the area before the commencement of research activity. The holder must obtain permission Prior Informed Consent (PIC) if collection is undertaken with or from the ethnic communities.

21. The permission is conditional upon the fulfilment of any requirement under the Immigration Act 1959/63 (Act 155) and the Immigration Regulations 1963, if any.*

22. The authority (Controller of Wild Life) has the right to impose any condition to the permit as it deems fit from time to time.

Thank you.

I CARE


OSWALD BRAKEN TISEN
for Controller of Wild Life and
National Parks & Nature Reserves
SARAWAK
nr/

"NEW FRONTIER IN BIODIVERSITY CONSERVATION"

SARAWAK FORESTRY Corporation
Lot 218, KCLD, Jalan Tapang, Kota Sentosa, 93250 Kuching
Tel: (+6) 082 610088 Fax: (+6) 082 610099 www.sarawakforestry.com

Figure 1: Permit approval by the SFC to conduct research on bats.

**Appendix C: Universiti Malaysia Sarawak Animal Ethics Committee (UNIMAS-AEC)
Ethics Approval**

Deputy Vice Chancellor's Office (Research and Innovation) Animal Ethics Committee (AEC) Tel: 082 581222/23 Fax: 082 665115	UNIVERSITI MALAYSIA SARAWAK 94300 Kota Samarahan
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MEMORANDUM

Reference : UNIMAS/TNC(PI)/09 – 65/03 (6)

To : Associate Professor Dr. Tan Cheng Siang
Faculty of Medicine & Health Sciences

From : Chairman
Animal Ethics Committee (AEC)

Date : 29 August 2022

Subject : Ethical Approval for Research

The Animal Ethics Committee (AEC) of Universiti Malaysia Sarawak (UNIMAS) has **approved** this study following the committee meeting on 23rd August 2022 (AEC Meeting 02/2022). The details of the research are as below:

Project : Investigating the Phylogenetic Diversity of SARS-CoV-like Viruses in Various Species of Bats

UNIMAS AEC Ref. No : UNIMAS/AEC/R/F07/074

Chief Investigator : Associate Professor Dr. Tan Cheng Siang
(Universiti Malaysia Sarawak)

Researchers : Associate Professor Dr. Mohd. Azlan Jayasilan bin Abdul Gulam Azad
(Universiti Malaysia Sarawak)

Associate Professor Dr. Faisal Ali bin Anwarali Khan
(Universiti Malaysia Sarawak)

Sultana Parvin binti Habeebur Rahman
(Universiti Malaysia Sarawak)

Thank you.

Yours sincerely,

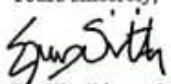

Prof Dr Edmund Sim Ui Hang
c.c: Deputy Vice Chancellor (Research & Innovation)
Director, UNIMAS Publisher

Figure 2: Research ethics approval by the UNIMAS-AEC to conduct this research.

Appendix D: Geographical Coordinates of Bat Sampling Sites in Sarawak.

Table 1: Sampling site coordinates for bat captures conducted across Sarawak

Region	Locality	Coordinate
Western	<i>Kampung Peros</i>	1.3635 °N, 110.1217 °E
	<i>Kampung Bobak</i>	1.5356 °N, 110.2005 °E
	<i>Kampung Gayu</i>	1.2172 °N, 110.3477 °E
	<i>Kampung Merdang Gayam</i>	1.4585 °N, 110.4102 °E
Northern	<i>Niah National Park</i>	3.8015 °N, 113.7853 °E
	<i>Mulu National Park</i>	4.0921 °N, 114.8958 °E

Appendix E: Bats Sampling Database

Table 2: The list of samples collected in this study with measurements.

Date	Field No.	Family	Identification (Genus/Species)	Sex	Measurements					
					Weight	Forearm	Tail	Ear	Tragus	Hindfoot
Sep-21	KPP3001	Pteropodidae	<i>Balionycteris maculata</i>	F	14.0	40.3	N/A	7.8	N/A	5.6
Sep-21	KPP4001	Pteropodidae	<i>Balionycteris maculata</i>	F	17.0	44.2	N/A	8.3	N/A	5.8
Sep-21	KPP2001	Pteropodidae	<i>Balionycteris maculata</i>	F	14.0	42.9	N/A	7.7	N/A	5.4
Sep-21	KPP2002	Pteropodidae	<i>Balionycteris maculata</i>	F	13.0	42.2	N/A	6.9	N/A	6.1
Sep-21	KPP4002	Pteropodidae	<i>Penthetor lucasi</i>	F	38.0	64.0	10.2	14.8	N/A	12.6
Sep-21	KPP4003	Pteropodidae	<i>Penthetor lucasi</i>	F	19.0	52.4	8.2	11.2	N/A	9.2

Table 2 continued

Sep-21	KPP4004	Pteropodidae	<i>Penthetor lucasi</i>	F	29.0	58.4	8.1	11.5	N/A	10.5
Sep-21	KPP4005	Pteropodidae	<i>Penthetor lucasi</i>	M	31.0	55.6	8.8	11.5	N/A	12.8
Sep-21	KPP4006	Pteropodidae	<i>Penthetor lucasi</i>	M	28.0	57.4	11.9	13.3	N/A	14.3
Sep-21	KPP2003	Pteropodidae	<i>Macroglossus minimus</i>	M	19.0	43.3	N/A	9.6	N/A	12.5
Sep-21	KPP2004	Pteropodidae	<i>Balionycteris maculata</i>	F	17.0	43.4	N/A	8.2	N/A	7.2
Sep-21	KPP2005	Pteropodidae	<i>Balionycteris maculata</i>	F	17.0	41.9	N/A	6.5	N/A	6.9
Sep-21	KPP2006	Pteropodidae	<i>Balionycteris maculata</i>	F	18.0	40.9	N/A	7.1	N/A	7.0
Sep-21	KPP1001	Pteropodidae	<i>Balionycteris maculata</i>	F	15.0	42.6	N/A	7.5	N/A	5.2

Table 2 continued

Sep-21	KPP1002	Pteropodidae	<i>Balionycteris maculata</i>	F	15.0	43.8	N/A	7.4	N/A	5.9
Sep-21	KPP4007	Pteropodidae	<i>Balionycteris maculata</i>	F	15.0	40.0	N/A	7.3	N/A	5.9
Sep-21	KPP3002	Pteropodidae	<i>Balionycteris maculata</i>	M	16.0	40.4	N/A	8.5	N/A	7.8
Sep-21	KPP4008	Pteropodidae	<i>Penthetor lucasi</i>	M	29.0	58.4	9.4	11.2	N/A	10.3
Sep-21	KPP4009	Pteropodidae	<i>Penthetor lucasi</i>	M	20.0	52.5	8.8	10.8	N/A	11.0
Sep-21	KPP2007	Pteropodidae	<i>Balionycteris maculata</i>	F	15.0	43.9	N/A	8.5	N/A	7.3
Sep-21	KPP2008	Pteropodidae	<i>Penthetor lucasi</i>	M	30.0	60.0	7.8	12.1	N/A	9.7
Sep-21	KPP5001	Pteropodidae	<i>Eonycteris spelaea</i>	M	52.0	65.4	14.1	13.9	N/A	15.6
Sep-21	KPP5002	Pteropodidae	<i>Penthetor lucasi</i>	M	29.0	55.3	9.4	12.7	N/A	9.3

Table 2 continued

Sep-21	KPP5003	Vespertilionidae	<i>Kerivoula hardwicki</i>	M	6.0	34.0	38.0	3.6	N/A	4.9
Sep-21	KPP5004	Pteropodidae	<i>Eonycteris spelaea</i>	M	63.0	68.8	12.0	17.3	N/A	15.5
Sep-21	KPP4010	Pteropodidae	<i>Balionycteris maculata</i>	F	16.0	42.3	N/A	8.4	N/A	9.1
Sep-21	KPP3003	Pteropodidae	<i>Penthetor lucasi</i>	M	23.0	55.1	8.1	12.1	N/A	11.1
Sep-21	KPP3004	Pteropodidae	<i>Penthetor lucasi</i>	M	32.0	58.9	9.5	14.0	N/A	12.5
Sep-21	KPP5005	Pteropodidae	<i>Macroglossus minimus</i>	M	18.0	42.4	N/A	9.7	N/A	9.7
Sep-21	KPP4011	Pteropodidae	<i>Balionycteris maculata</i>	F	17.0	42.7	N/A	7.8	N/A	8.4
Sep-21	KPP4012	Pteropodidae	<i>Balionycteris maculata</i>	M	16.0	38.9	N/A	8.1	N/A	6.9

Table 2 continued

Sep-21	KPP4013	Pteropodidae	<i>Balionycteris maculata</i>	M	15.0	43.2	N/A	9.4	N/A	6.1
Oct-21	KBP1001	Pteropodidae	<i>Cynopterus brachyotis</i>	F	29.0	59.0	9.5	13.7	N/A	9.0
Oct-21	KBP1002	Pteropodidae	<i>Cynopterus brachyotis</i>	M	31.0	60.0	14.4	10.1	N/A	9.9
Oct-21	KBP2001	Pteropodidae	<i>Balionycteris maculata</i>	F	12.0	41.9	N/A	8.6	N/A	5.2
Oct-21	KBP3001	Pteropodidae	<i>Dycopterus spadiceus</i>	M	55.0	65.7	15.2	15.0	N/A	9.7
Oct-21	KBP3002	Pteropodidae	<i>Dycopterus spadiceus</i>	M	64.0	66.7	12.4	16.7	N/A	13.2
Oct-21	KBP3003	Pteropodidae	<i>Dycopterus spadiceus</i>	M	59.0	70.0	14.6	16.9	N/A	14.5

Table 2 continued

Oct-21	KBP4001	Pteropodidae	<i>Cynopterus brachyotis</i>	F	36.0	65.5	12.9	15.7	N/A	11.5
Oct-21	KBP5001	Pteropodidae	<i>Penthetor lucasi</i>	F	51.0	64.6	13.6	15.9	N/A	13.3
Oct-21	KBP5003	Pteropodidae	<i>Cynopterus brachyotis</i>	F	33.0	60.5	9.2	15.6	N/A	9.6
Oct-21	KBP5004	Pteropodidae	<i>Cynopterus brachyotis</i>	M	33.0	60.5	8.6	10.1	N/A	8.2
Oct-21	KBP5005	Pteropodidae	<i>Dycopterus spadiceus</i>	F	40.0	62.0	13.7	13.2	N/A	16.8
Oct-21	KBP5006	Pteropodidae	<i>Penthetor lucasi</i>	F	35.0	63.8	11.9	13.3	N/A	12.7
Oct-21	KBP5007	Pteropodidae	<i>Penthetor lucasi</i>	F	33.0	62.7	8.5	12.9	N/A	9.2
Oct-21	KBP5008	Pteropodidae	<i>Penthetor lucasi</i>	M	36.0	62.3	9.4	10.2	N/A	9.0
Oct-21	KBP5009	Pteropodidae	<i>Cynopterus brachyotis</i>	F	49.0	63.8	16.1	12.2	N/A	11.1

Table 2 continued

Oct-21	KBP5010	Pteropodidae	<i>Cynopterus brachyotis</i>	M	38.0	63.0	10.3	13.1	N/A	11.6
Oct-21	KBP5011	Pteropodidae	<i>Dycopterus spadiceus</i>	F	53.0	67.5	11.1	12.7	N/A	11.3
Oct-21	KBP5012	Rhinolophidae	<i>Rhinolophus luctus</i>	M	29.0	64.8	45.5	29.9	N/A	9.6
Oct-21	KBP3004	Pteropodidae	<i>Cynopterus brachyotis</i>	M	30.0	60.9	11.2	14.5	N/A	7.9
Oct-21	KBP3005	Pteropodidae	<i>Cynopterus brachyotis</i>	M	28.0	61.8	10.5	13.3	N/A	9.2
Dec-21	KPG 001	Pteropodidae	<i>Penthetor lucasi</i>	F	40.0	60.8	11.0	14.0	N/A	9.3
Dec-21	KPG 002	Pteropodidae	<i>Eonycteris spelaea</i>	M	49.0	64.6	15.6	14.7	N/A	N/A
Dec-21	KPG 003	Pteropodidae	<i>Penthetor lucasi</i>	F	N/A	64.5	N/A	N/A	N/A	N/A
Dec-21	KPG 004	Pteropodidae	<i>Penthetor lucasi</i>	M	42.0	61.2	13.8	16.2	N/A	13.2
Dec-21	KPG 005	Pteropodidae	<i>Eonycteris spelaea</i>	F	55.0	67.1	14.9	15.5	N/A	11.8

Table 2 continued

Dec-21	KPG 006	Pteropodidae	<i>Penthetor lucasi</i>	M	39.0	57.7	9.6	13.0	N/A	11.4
Dec-21	KPG 007	Pteropodidae	<i>Penthetor lucasi</i>	M	N/A	58.5	11.5	12.9	N/A	9.8
Dec-21	KPG 008	Pteropodidae	<i>Cynopterus brachyotis</i>	M	N/A	59.7	12.8	11.5	N/A	8.4
Dec-21	KPG 009	Pteropodidae	<i>Penthetor lucasi</i>	M	N/A	57.9	10.5	13.5	N/A	10.9
Dec-21	KPG 010	Pteropodidae	<i>Penthetor lucasi</i>	F	51.0	68.4	15.2	15.5	N/A	11.0
Dec-21	KPG 012	Pteropodidae	<i>Eonycteris spelaea</i>	M	57.0	64.7	12.0	15.1	N/A	12.4
Dec-21	KPG 013	Pteropodidae	<i>Eonycteris spelaea</i>	F	49.0	65.4	15.9	14.1	N/A	8.9
Dec-21	KPG 014	Pteropodidae	<i>Eonycteris spelaea</i>	F	72.0	84.9	13.9	15.9	N/A	13.9
Dec-21	KPG 015	Pteropodidae	<i>Eonycteris spelaea</i>	F	N/A	54.9	9.0	12.9	N/A	11.7
Dec-21	KPG 016	Vespertilionidae	<i>Kerivoula papillosa</i>	F	28.0	39.5	44.9	9.9	N/A	7.9
Dec-21	KPG 017	Pteropodidae	<i>Macroglossus minimus</i>	M	11.0	37.2	N/A	10.5	N/A	5.4
Dec-21	KPG 018	Pteropodidae	<i>Eonycteris spelaea</i>	M	47.0	75.9	14.8	16.8	N/A	14.7

Table 2 continued

Dec-21	KPG 019	Pteropodidae	<i>Penthetor lucasi</i>	F	40.0	63.2	12.5	11.8	N/A	11.4
Dec-21	KPG 020	Pteropodidae	<i>Penthetor lucasi</i>	M	31.0	59.0	8.3	15.1	N/A	8.5
Dec-21	KPG 021	Pteropodidae	<i>Penthetor lucasi</i>	M	36.0	58.4	12.1	16.2	N/A	11.7
Dec-21	KPG 022	Pteropodidae	<i>Eonycteris spelaea</i>	F	40.0	63.1	14.2	14.4	N/A	12.2
Jan-22	MG001	Pteropodidae	<i>Cynopterus brachyotis</i>	F	32.0	62.3	11.7	14.3	N/A	9.6
Jan-22	MG002	Pteropodidae	<i>Cynopterus brachyotis</i>	F	38.0	65.8	15.1	12.7	N/A	11.6
Jan-22	MG003	Pteropodidae	<i>Cynopterus brachyotis</i>	F	30.0	65.3	12.5	14.4	N/A	8.5
Jan-22	MG004	Pteropodidae	<i>Cynopterus brachyotis</i>	F	33.0	65.7	14.5	15.3	N/A	10.6
Jan-22	MG005	Pteropodidae	<i>Cynopterus brachyotis</i>	F	33.0	63.6	13.4	16.2	N/A	8.7

Table 2 continued

Jan-22	MG006	Pteropodidae	<i>Cynopterus brachyotis</i>	F	33.0	65.9	13.8	13.3	N/A	8.9
Jan-22	MG007	Pteropodidae	<i>Cynopterus horsfieldi</i>	M	43.0	69.9	16.1	13.8	N/A	11.6
Jan-22	MG008	Pteropodidae	<i>Cynopterus brachyotis</i>	M	31.0	63.5	11.1	16.1	N/A	8.4
Jan-22	MG009	Pteropodidae	<i>Cynopterus brachyotis</i>	M	N/A	55.1	10.7	15.2	N/A	8.9
Jan-22	MG010	Pteropodidae	<i>Cynopterus brachyotis</i>	F	45.0	65.5	12.9	14.1	N/A	8.3
Jan-22	MG011	Pteropodidae	<i>Cynopterus brachyotis</i>	M	28.0	65.9	13.2	15.9	N/A	10.0
Jan-22	MG012	Pteropodidae	<i>Cynopterus brachyotis</i>	F	43.0	65.0	11.8	14.9	N/A	9.7

Table 2 continued

Jan-22	MG013	Pteropodidae	<i>Cynopterus brachyotis</i>	F	34.0	63.4	14.2	14.3	N/A	10.2
Jan-22	MG014	Pteropodidae	<i>Cynopterus brachyotis</i>	F	33.0	67.6	16.3	16.3	N/A	9.6
Jan-22	MG015	Pteropodidae	<i>Dycopterus spadiceus</i>	M	66.0	68.0	18.2	16.9	N/A	12.6
Jan-22	MG016	Pteropodidae	<i>Dycopterus spadiceus</i>	M	48.0	61.4	19.2	16.8	N/A	14.0
Jan-22	MG017	Pteropodidae	<i>Dycopterus spadiceus</i>	F	55.0	68.3	14.6	14.1	N/A	11.1
Jan-22	MG018	Pteropodidae	<i>Penthetor lucasi</i>	M	35.0	57.1	14.9	16.1	N/A	9.8
Jan-22	MG019	Pteropodidae	<i>Macroglossus minimus</i>	F	16.0	41.5	N/A	13.4	N/A	8.8

Table 2 continued

Jan-22	MG020	Pteropodidae	<i>Macroglossus minimus</i>	F	16.0	40.1	N/A	12.9	N/A	6.5
Jan-22	MG021	Pteropodidae	<i>Cynopterus brachyotis</i>	F	39.0	66.1	15.1	15.4	N/A	7.7
Jan-22	MG022	Pteropodidae	<i>Penthetor lucasi</i>	M	40.0	62.1	16.0	14.5	N/A	11.7
Jan-22	MG023	Pteropodidae	<i>Cynopterus brachyotis</i>	M	24.0	55.8	11.7	11.8	N/A	10.6
Jan-22	MG024	Pteropodidae	<i>Penthetor lucasi</i>	F	55.0	64.3	15.3	17.2	N/A	14.1
Jan-22	MG025	Pteropodidae	<i>Cynopterus brachyotis</i>	M	36.0	65.3	11.4	14.7	N/A	7.3
Jan-22	MG026	Pteropodidae	<i>Cynopterus brachyotis</i>	M	34.0	65.4	13.3	15.1	N/A	13.7
Jan-22	MG027	Pteropodidae	<i>Cynopterus brachyotis</i>	M	27.0	58.5	15.1	13.6	N/A	10.1

Table 2 continued

Jan-22	MG028	Pteropodidae	<i>Cynopterus brachyotis</i>	F	33.0	60.0	17.8	14.7	N/A	12.1
Jan-22	MG029	Pteropodidae	<i>Cynopterus brachyotis</i>	M	31.0	60.9	11.6	12.7	N/A	9.8
Jan-22	MG030	Pteropodidae	<i>Penthetor lucasi</i>	M	43.0	60.9	18.1	16.1	N/A	14.0
Jan-22	MG031	Pteropodidae	<i>Cynopterus brachyotis</i>	M	28.0	62.9	12.2	16.9	N/A	6.6
Jan-22	MG032	Pteropodidae	<i>Cynopterus brachyotis</i>	M	38.0	63.0	13.5	16.1	N/A	11.5
Jan-22	MG033	Pteropodidae	<i>Cynopterus brachyotis</i>	M	30.0	64.2	10.2	14.0	N/A	8.2
Jan-22	MG034	Pteropodidae	<i>Cynopterus brachyotis</i>	M	38.0	63.0	18.4	15.1	N/A	13.5

Table 2 continued

Jan-22	MG035	Pteropodidae	<i>Cynopterus brachyotis</i>	M	21.0	55.1	10.0	13.6	N/A	6.7
Jan-22	MG036	Pteropodidae	<i>Cynopterus brachyotis</i>	M	33.0	61.1	15.4	14.3	N/A	13.8
Jan-22	MG037	Pteropodidae	<i>Cynopterus brachyotis</i>	M	25.0	60.4	11.5	13.3	N/A	7.8
Jan-22	MG038	Pteropodidae	<i>Cynopterus brachyotis</i>	F	45.0	65.1	16.5	13.5	N/A	12.8
Jan-22	MG039	Pteropodidae	<i>Cynopterus horsfieldi</i>	M	41.0	65.7	13.5	13.7	N/A	9.5
Jan-22	MG040	Pteropodidae	<i>Macroglossus minimus</i>	M	14.0	40.2	N/A	14.3	N/A	8.7
Jan-22	MG041	Pteropodidae	<i>Macroglossus minimus</i>	F	16.0	43.1	N/A	13.1	N/A	5.6

Table 2 continued

Jan-22	MG042	Pteropodidae	<i>Cynopterus brachyotis</i>	M	33.0	62.7	11.6	14.2	N/A	12.4
Jan-22	MG043	Pteropodidae	<i>Macroglossus minimus</i>	M	12.0	39.4	N/A	12.1	N/A	7.0
Jan-22	MG044	Pteropodidae	<i>Cynopterus brachyotis</i>	F	22.0	59.0	10.8	12.8	N/A	11.5
Jan-22	MG045	Pteropodidae	<i>Penthetor lucasi</i>	M	38.0	58.9	15.5	16.1	N/A	14.1
Jan-22	MG046	Pteropodidae	<i>Cynopterus brachyotis</i>	M	38.0	63.3	14.1	14.3	N/A	8.1
Jan-22	MG047	Pteropodidae	<i>Cynopterus horsfieldi</i>	F	45.0	64.9	15.1	15.0	N/A	7.0
Jan-22	MG048	Pteropodidae	<i>Cynopterus brachyotis</i>	F	23.0	59.4	12.8	14.1	N/A	12.4

Table 2 continued

Jan-22	MG049	Pteropodidae	<i>Cynopterus brachyotis</i>	F	43.0	63.6	15.1	14.9	N/A	11.9
Jan-22	MG050	Pteropodidae	<i>Macroglossus minimus</i>	F	16.0	42.0	N/A	12.6	N/A	6.9
Jan-22	MG051	Pteropodidae	<i>Cynopterus brachyotis</i>	M	25.0	61.6	11.5	16.1	N/A	9.8
Jan-22	MG052	Pteropodidae	<i>Macroglossus minimus</i>	M	14.0	41.5	N/A	14.1	N/A	6.0
Jan-22	MG053	Pteropodidae	<i>Cynopterus brachyotis</i>	F	28.0	60.8	12.5	14.4	N/A	10.1
Jan-22	MG054	Pteropodidae	<i>Cynopterus brachyotis</i>	F	36.0	65.5	12.4	14.2	N/A	12.7
Jan-22	MG055	Pteropodidae	<i>Cynopterus brachyotis</i>	M	22.0	60.0	10.0	12.4	N/A	7.6

Table 2 continued

Jan-22	MG056	Pteropodidae	<i>Cynopterus brachyotis</i>	F	30.0	60.0	12.1	14.5	N/A	11.7
Jan-22	MG057	Pteropodidae	<i>Cynopterus brachyotis</i>	F	35.0	65.3	8.1	13.8	N/A	10.0
Jan-22	MG058	Pteropodidae	<i>Penthetor lucasi</i>	M	57.0	63.6	14.9	13.9	N/A	10.0
Jan-22	MG059	Pteropodidae	<i>Cynopterus brachyotis</i>	M	26.0	63.5	11.4	11.4	N/A	8.9
Jan-22	MG060	Pteropodidae	<i>Cynopterus brachyotis</i>	M	32.0	65.7	11.5	14.6	N/A	7.6
Jan-22	MG061	Pteropodidae	<i>Macroglossus minimus</i>	M	14.0	41.0	N/A	12.0	N/A	9.0
Jan-22	MG062	Pteropodidae	<i>Cynopterus horsfieldi</i>	M	41.0	64.9	9.8	13.2	N/A	13.8

Table 2 continued

Jan-22	MG063	Pteropodidae	<i>Cynopterus horsfieldi</i>	M	33.0	66.0	14.0	10.5	N/A	13.0
Jan-22	MG064	Pteropodidae	<i>Penthetor lucasi</i>	M	27.0	55.0	14.0	11.5	N/A	13.5
Jan-22	MG065	Pteropodidae	<i>Cynopterus brachyotis</i>	M	33.0	60.7	12.0	15.0	N/A	8.8
Jan-22	MG066	Pteropodidae	<i>Cynopterus brachyotis</i>	F	23.0	61.5	12.0	10.5	N/A	12.5
Jan-22	MG067	Pteropodidae	<i>Cynopterus brachyotis</i>	M	33.0	61.3	12.7	15.3	N/A	7.3
Jan-22	MG068	Pteropodidae	<i>Macroglossus minimus</i>	M	16.0	42.2	N/A	12.8	N/A	7.2
Jan-22	MG069	Pteropodidae	<i>Cynopterus brachyotis</i>	F	32.0	67.0	14.5	15.5	N/A	12.0

Table 2 continued

Jan-22	MG070	Pteropodidae	<i>Cynopterus horsfieldi</i>	M	39.0	63.2	12.0	13.4	N/A	8.1
Nov-22	NNP22-015	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	8.0	44.2	17.9	16.3	N/A	8.2
Nov-22	NNP22-016	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	8.0	42.0	20.0	15.6	N/A	N/A
Nov-22	NNP22-017	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	16.0	41.0	20.3	15.2	N/A	N/A
Nov-22	NNP22-018	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	9.0	43.8	24.3	16.6	N/A	N/A
Nov-22	NNP22-019	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	7.0	43.6	21.1	13.6	N/A	N/A
Nov-22	NNP22-020	Vespertilionidae	<i>Kerivoula pellucida</i>	M	7.0	30.8	44.6	14.2	8.1	N/A

Table 2 continued

Nov-22	NNP22-012	Rhinolophidae	<i>Rhinolophus philippinensis</i>	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nov-22	NNP22-021	Hipposideridae	<i>Hipposideros cervinus</i>	M	6.0	43.4	23.3	11.5	N/A	N/A
Nov-22	NNP22-022	Hipposideridae	<i>Hipposideros cervinus</i>	M	6.0	45.5	23.8	12.7	N/A	N/A
Nov-22	NNP22-023	Hipposideridae	<i>Hipposideros cervinus</i>	F	8.0	51.1	37.9	15.3	N/A	N/A
Nov-22	NNP22-024	Hipposideridae	<i>Hipposideros cervinus</i>	F	9.0	51.6	38.2	13.3	N/A	N/A
Nov-22	NNP22-025	Hipposideridae	<i>Hipposideros cervinus</i>	M	8.0	47.3	24.7	10.8	N/A	N/A
Nov-22	NNP22-026	Hipposideridae	<i>Hipposideros ater</i>	F	5.0	41.2	20.1	16.8	N/A	N/A

Table 2 continued

Nov-22	NNP22-027	Pteropodidae	<i>Cynopterus brachyotis</i>	M	17.0	57.3	8.6	13.9	N/A	N/A
Nov-22	NNP22-028	Pteropodidae	<i>Cynopterus brachyotis</i>	M	26.0	57.4	10.6	12.3	N/A	N/A
Nov-22	NNP22-029	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	10.0	54.1	25.3	26.3	N/A	N/A
Nov-22	NNP22-030	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	N/A	53.8	24.4	28.9	N/A	N/A
Nov-22	NNP22-031	Pteropodidae	<i>Cynopterus brachyotis</i>	F	4.0	55.0	9.6	12.9	N/A	N/A
Nov-22	NNP22-032	Pteropodidae	<i>Cynopterus brachyotis</i>	F	N/A	55.1	11.9	11.8	N/A	N/A
Nov-22	NNP22-033	Pteropodidae	<i>Cynopterus brachyotis</i>	F	25.0	55.5	12.0	13.9	N/A	N/A

Table 2 continued

Nov-22	NNP22-034	Hipposideridae	<i>Hipposideros diadema</i>	F	39.0	82.1	43.7	25.1	N/A	14.5
Nov-22	NNP22-035	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	9.0	52.4	26.4	27.4	N/A	6.5
Nov-22	NNP22-036	Pteropodidae	<i>Cynopterus brachyotis</i>	M	25.0	58.1	10.3	11.0	N/A	8.7
Nov-22	NNP22-037	Pteropodidae	<i>Cynopterus brachyotis</i>	F	31.0	57.6	10.0	13.0	N/A	10.0
Nov-22	NNP22-038	Hipposideridae	<i>Hipposideros ater</i>	M	7.0	43.8	25.8	11.0	N/A	7.7
Nov-22	NNP22-039	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	10.0	41.3	20.7	15.0	N/A	7.3
Nov-22	NNP22-040	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	11.0	52.1	26.7	28.8	N/A	8.9

Table 2 continued

Nov-22	NNP22-041	Nycteridae	<i>Nycteris tragata</i>	F	13.0	53.5	63.7	26.4	N/A	6.9
Nov-22	NNP22-042	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	9.0	45.1	24.5	13.4	N/A	8.0
Nov-22	NNP22-043	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	8.0	42.7	23.2	14.3	N/A	6.8
Nov-22	NNP22-044	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	8.0	50.6	27.9	28.7	N/A	7.4
Nov-22	NNP22-050	Vespertilionidae	<i>Murina suilla</i>	M	4.0	30.4	30.0	10.5	6.1	7.6
Nov-22	NNP22-051	Pteropodidae	<i>Balionycteris maculata</i>	F	13.0	41.6	8.3	N/A	N/A	7.9
Nov-22	NNP22-052	Pteropodidae	<i>Penthetor lucasi</i>	F	33.0	59.8	8.3	13.9	N/A	13.4

Table 2 continued

Nov-22	NNP22-053	Vespertilionidae	<i>Myotis gomantongensis</i>	F	12.0	43.5	37.3	10.8	N/A	8.1
Nov-22	NNP22-054	Vespertilionidae	<i>Myotis gomantongensis</i>	F	7.0	43.4	39.2	12.4	4.1	8.4
Nov-22	NNP22-055	Pteropodidae	<i>Balionycteris maculata</i>	M	N/A	40.6	N/A	8.0	N/A	4.5
Nov-22	NNP22-056	Vespertilionidae	<i>Myotis cf. horsfieldi</i>	F	8.0	36.8	35.5	11.1	5.3	10.1
Nov-22	NNP22-057	Miniopteridae	<i>Miniopterus magnater</i>	F	17.0	48.6	54.2	10.1	3.4	5.9
Nov-22	NNP22-058	Vespertilionidae	<i>Myotis cf. horsfieldi</i>	M	10.0	38.2	34.2	11.3	3.4	7.9
Nov-22	NNP22-060	Miniopteridae	<i>Miniopterus magnater</i>	F	15.0	48.5	51.4	11.2	4.8	8.0

Table 2 continued

Nov-22	NNP22-061	Miniopteridae	<i>Miniopterus magnater</i>	M	10.0	49.3	59.3	11.0	3.6	6.2
Nov-22	NNP22-062	Pteropodidae	<i>Penthetor lucasi</i>	F	30.0	60.9	12.4	15.3	N/A	11.2
Nov-22	NNP22-063	Pteropodidae	<i>Balionycteris maculata</i>	M	13.0	39.8	N/A	9.3	N/A	6.6
Nov-22	NNP22-064	Miniopteridae	<i>Miniopterus magnater</i>	M	10.0	50.9	55.0	11.0	3.5	7.0
Nov-22	NNP22-065	Vespertilionidae	<i>Myotis gomantongensis</i>	F	10.0	42.0	31.3	11.0	6.0	9.0
Nov-22	NNP22-066	Hipposideridae	<i>Hipposideros diadema</i>	M	40.0	83.8	52.6	21.0	N/A	13.2
Nov-22	NNP22-067	Vespertilionidae	<i>Murina suilla</i>	M	5.0	30.6	27.4	10.1	5.3	7.1

Table 2 continued

Nov-22	NNP22-068	Rhinolophidae	<i>Rhinolophus trifolius</i>	M	10.0	51.3	27.4	21.6	N/A	8.4
Nov-22	NNP22-069	Vespertilionidae	<i>Myotis horsfieldi</i>	F	7.0	37.5	34.2	12.2	4.9	8.3
Jun-23	MNP001	Pteropodidae	<i>Balionycteris maculata</i>	F	13.0	41.5	N/A	8.4	N/A	4.7
Jun-23	MNP002	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	11.0	46.5	N/A	9.9	N/A	7.0
Jun-23	MNP003	Hipposideridae	<i>Hipposideros galeritus</i>	M	7.0	48.5	30.7	11.9	N/A	4.4
Jun-23	MNP004	Hipposideridae	<i>Hipposideros galeritus</i>	F	9.0	47.1	33.2	10.9	N/A	6.6
Jun-23	MNP005	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	45.3	N/A	13.0	N/A	6.9

Table 2 continued

Jun-23	MNP006	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	46.6	35.4	9.3	N/A	5.5
Jun-23	MNP007	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	47.7	33.2	10.6	N/A	4.4
Jun-23	MNP008	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	45.3	21.0	19.3	N/A	6.2
Jun-23	MNP009	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	21.0	53.9	N/A	32.2	16.6	12.6
Jun-23	MNP010	Hipposideridae	<i>Hipposideros cervinus</i>	M	9.0	45.7	25.2	10.3	N/A	4.5
Jun-23	MNP011	Hipposideridae	<i>Hipposideros cervinus</i>	F	9.0	46.1	22.1	11.6	N/A	4.7
Jun-23	MNP012	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	46.0	34.5	9.8	N/A	4.6

Table 2 continued

Jun-23	MNP013	Hipposideridae	<i>Hipposideros galeritus</i>	F	9.0	46.9	32.7	9.6	N/A	4.5
Jun-23	MNP014	Vespertilionidae	<i>Kerivoula papillosa</i>	M	7.0	46.0	47.0	13.9	9.5	7.2
Jun-23	MNP015	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	11.0	47.4	N/A	15.8	N/A	6.2
Jun-23	MNP016	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	12.0	46.9	N/A	15.2	N/A	8.2
Jun-23	MNP017	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	48.0	N/A	15.3	N/A	6.9
Jun-23	MNP019	Hipposideridae	<i>Hipposideros cervinus</i>	M	11.0	47.9	22.6	11.0	N/A	5.6
Jun-23	MNP020	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	12.0	48.1	N/A	16.5	N/A	7.4

Table 2 continued

Jun-23	MNP021	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	14.0	47.5	59.3	25.4	N/A	7.1
Jun-23	MNP022	Hipposideridae	<i>Hipposideros galeritus</i>	M	9.0	48.3	31.5	10.1	N/A	4.5
Jun-23	MNP023	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	12.0	47.7	N/A	10.8	N/A	7.7
Jun-23	MNP024	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	13.0	46.5	N/A	17.6	N/A	8.3
Jun-23	MNP025	Vespertilionidae	<i>Kerivoula hardwicki</i>	M	3.0	32.2	40.0	11.6	5.8	5.6
Jun-23	MNP026	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	13.0	46.4	N/A	14.3	N/A	7.6
Jun-23	MNP027	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	8.0	45.4	N/A	18.0	N/A	7.9

Table 2 continued

Jun-23	MNP028	Hipposideridae	<i>Hipposideros galeritus</i>	F	9.0	46.7	33.2	9.0	N/A	4.7
Jun-23	MNP029	Hipposideridae	<i>Hipposideros cervinus</i>	F	9.0	48.4	27.3	10.9	N/A	6.6
Jun-23	MNP030	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	11.0	46.7	N/A	15.9	N/A	6.9
Jun-23	MNP031	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	6.0	39.2	N/A	14.6	N/A	5.0
Jun-23	MNP032	Hipposideridae	<i>Hipposideros galeritus</i>	M	10.0	45.0	33.9	10.0	N/A	5.3
Jun-23	MNP033	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	11.0	47.3	N/A	17.2	N/A	6.7
Jun-23	MNP034	Hipposideridae	<i>Hipposideros coxi</i>	M	8.0	51.9	25.6	17.1	N/A	3.7

Table 2 continued

Jun-23	MNP035	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	46.7	32.0	10.9	N/A	4.1
Jun-23	MNP036	Hipposideridae	<i>Hipposideros galeritus</i>	F	9.0	46.0	30.2	11.4	N/A	4.1
Jun-23	MNP037	Hipposideridae	<i>Hipposideros galeritus</i>	M	7.0	46.7	31.8	11.7	N/A	6.1
Jun-23	MNP038	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	46.6	N/A	18.0	N/A	8.5
Jun-23	MNP039	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	7.0	47.4	N/A	16.0	N/A	7.2
Jun-23	MNP040	Hipposideridae	<i>Hipposideros cervinus</i>	M	9.0	46.4	18.0	15.1	N/A	5.2
Jun-23	MNP041	Hipposideridae	<i>Hipposideros cervinus</i>	M	8.0	46.4	20.0	12.0	N/A	5.4

Table 2 continued

Jun-23	MNP042	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	9.0	43.7	N/A	16.4	N/A	8.8
Jun-23	MNP043	Hipposideridae	<i>Hipposideros galeritus</i>	M	9.0	46.7	31.9	13.1	N/A	4.5
Jun-23	MNP044	Vespertilionidae	<i>Kerivoula minuta</i>	M	3.0	28.2	31.6	12.0	7.2	4.0
Jun-23	MNP045	Hipposideridae	<i>Hipposideros galeritus</i>	F	9.0	45.0	32.1	11.3	N/A	5.8
Jun-23	MNP046	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	9.0	43.0	22.0	15.5	N/A	5.9
Jun-23	MNP047	Hipposideridae	<i>Hipposideros cervinus</i>	M	8.0	44.4	22.0	14.8	N/A	5.6
Jun-23	MNP048	Hipposideridae	<i>Hipposideros cervinus</i>	F	6.0	45.1	21.0	14.9	N/A	5.4

Table 2 continued

Jun-23	MNP049	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	6.0	40.6	N/A	10.1	N/A	4.3
Jun-23	MNP050	Hipposideridae	<i>Hipposideros cervinus</i>	M	14.0	47.9	22.0	13.6	N/A	5.0
Jun-23	MNP051	Hipposideridae	<i>Hipposideros galeritus</i>	M	6.0	46.3	31.5	10.1	N/A	5.6
Jun-23	MNP052	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	7.0	44.7	19.3	14.9	N/A	5.0
Jun-23	MNP053	Hipposideridae	<i>Hipposideros cervinus</i>	M	8.0	43.1	21.7	13.2	N/A	5.1
Jun-23	MNP054	Hipposideridae	<i>Hipposideros galeritus</i>	M	7.0	46.0	31.0	14.3	N/A	4.0
Jun-23	MNP055	Pteropodidae	<i>Cynopterus brachyotis</i>	F	N/A	N/A	N/A	N/A	N/A	N/A

Table 2 continued

Jun-23	MNP056	Hipposideridae	<i>Hipposideros cervinus</i>	M	9.0	46.5	22.0	12.8	N/A	6.9
Jun-23	MNP057	Hipposideridae	<i>Hipposideros cervinus</i>	F	5.0	44.8	21.0	12.9	N/A	4.8
Jun-23	MNP058	Hipposideridae	<i>Hipposideros galeritus</i>	M	6.0	45.6	30.7	12.9	N/A	5.2
Jun-23	MNP059	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	7.0	40.8	N/A	16.9	N/A	5.4
Jun-23	MNP060	Hipposideridae	<i>Hipposideros galeritus</i>	M	6.0	45.6	31.9	12.0	N/A	3.7
Jun-23	MNP061	Hipposideridae	<i>Hipposideros cervinus</i>	F	8.0	46.2	22.0	16.0	N/A	4.9
Jun-23	MNP062	Vespertilionidae	<i>Murina sp.</i>	M	9.0	36.6	26.0	10.8	4.5	7.2

Table 2 continued

Jun-23	MNP063	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	9.0	48.9	N/A	18.5	N/A	6.0
Jun-23	MNP064	Pteropodidae	<i>Cynopterus brachyotis</i>	M	23.0	N/A	N/A	N/A	N/A	N/A
Jun-23	MNP065	Hipposideridae	<i>Hipposideros cervinus</i>	F	9.0	46.9	23.0	12.9	N/A	6.3
Jun-23	MNP066	Vespertilionidae	<i>Murina sp.</i>	M	6.0	34.0	23.0	10.8	6.1	7.4
Jun-23	MNP067	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	5.0	49.2	N/A	18.4	N/A	7.9
Jun-23	MNP068	Hipposideridae	<i>Hipposideros cervinus</i>	M	6.0	34.8	19.6	16.3	N/A	5.2
Jun-23	MNP069	Hipposideridae	<i>Hipposideros galeritus</i>	M	7.0	46.9	30.1	10.7	N/A	6.1

Table 2 continued

Jun-23	MNP070	Hipposideridae	<i>Hipposideros</i> <i>diadema</i>	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Jun-23	MNP071	Hipposideridae	<i>Hipposideros</i> <i>diadema</i>	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Jun-23	MNP072	Hipposideridae	<i>Hipposideros</i> <i>diadema</i>	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Jun-23	MNP073	Rhinolophidae	<i>Rhinolophus</i> <i>borneensis</i>	F	9.0	46.6	14.1	16.7	N/A	8.1
Jun-23	MNP074	Hipposideridae	<i>Hipposideros</i> <i>galeritus</i>	M	7.0	44.6	32.2	11.5	N/A	4.3
Jun-23	MNP075	Rhinolophidae	<i>Rhinolophus</i> <i>borneensis</i>	F	9.0	40.1	13.2	14.2	N/A	6.5
Jun-23	MNP076	Rhinolophidae	<i>Rhinolophus</i> <i>borneensis</i>	M	7.0	41.6	19.0	15.6	N/A	7.1

Table 2 continued

Jun-23	MNP077	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	7.0	40.7	17.6	15.6	N/A	6.2
Jun-23	MNP078	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	6.0	40.5	19.5	15.7	N/A	7.0
Jun-23	MNP079	Nycteridae	<i>Nycteris tragata</i>	M	15.0	45.4	62.3	27.3	N/A	9.3
Jun-23	MNP080	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	9.0	47.3	21.0	16.8	N/A	8.9
Jun-23	MNP081	Hipposideridae	<i>Hipposideros coxi</i>	M	9.0	46.6	28.2	19.5	N/A	6.9
Jun-23	MNP082	Hipposideridae	<i>Hipposideros cervinus</i>	M	8.0	47.6	22.9	12.1	N/A	6.2
Jun-23	MNP083	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	14.0	45.6	17.9	17.0	N/A	9.4
Jun-23	MNP084	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	7.0	48.7	N/A	17.8	N/A	8.6

Table 2 continued

Jun-23	MNP085	Nycteridae	<i>Nycteris tragata</i>	M	14.0	48.3	60.0	22.9	N/A	9.6
Jun-23	MNP086	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	46.6	N/A	18.5	N/A	8.1
Jun-23	MNP087	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	46.6	31.2	13.2	N/A	5.0
Jun-23	MNP088	Hipposideridae	<i>Hipposideros dyacorum</i>	F	5.0	39.0	N/A	14.7	N/A	6.0
Jun-23	MNP089	Hipposideridae	<i>Hipposideros galeritus</i>	F	7.0	46.3	36.0	12.0	N/A	5.0
Jun-23	MNP090	Hipposideridae	<i>Hipposideros cervinus</i>	M	8.0	44.4	19.3	13.4	N/A	6.8
Jun-23	MNP091	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	45.3	N/A	16.2	N/A	7.2

Table 2 continued

Jun-23	MNP092	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	47.3	N/A	15.9	N/A	9.6
Jun-23	MNP093	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	7.0	42.7	19.5	14.9	N/A	6.5
Jun-23	MNP094	Hipposideridae	<i>Hipposideros cervinus</i>	M	10.0	47.4	22.8	15.4	N/A	5.9
Jun-23	MNP095	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	N/A	N/A	N/A	N/A	N/A	N/A
Jun-23	MNP096	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	10.0	50.5	25.8	25.4	N/A	7.2
Jun-23	MNP097	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	15.0	47.3	25.4	25.7	N/A	7.4
Jun-23	MNP098	Hipposideridae	<i>Hipposideros dyacorum</i>	M	7.0	39.3	19.7	15.7	N/A	6.2

Table 2 continued

Jun-23	MNP099	Hipposideridae	<i>Hipposideros galeritus</i>	F	6.0	48.7	30.8	11.7	N/A	6.1
Jun-23	MNP100	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	49.7	N/A	19.0	N/A	7.2
Jun-23	MNP101	Hipposideridae	<i>Hipposideros bicolor</i>	M	9.0	46.0	28.1	16.9	N/A	5.1
Jun-23	MNP102	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	5.0	39.9	19.7	13.7	N/A	5.2
Jun-23	MNP103	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	6.0	40.6	18.6	14.3	N/A	5.6
Jun-23	MNP104	Hipposideridae	<i>Hipposideros bicolor</i>	M	8.0	45.4	31.1	18.9	N/A	5.7
Jun-23	MNP105	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	49.7	33.3	11.4	N/A	6.5

Table 2 continued

Jun-23	MNP106	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	8.0	42.0	18.5	12.8	N/A	6.2
Jun-23	MNP107	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	7.0	40.0	17.9	16.9	N/A	6.0
Jun-23	MNP108	Hipposideridae	<i>Hipposideros galeritus</i>	F	7.0	46.2	30.2	12.3	N/A	5.3
Jun-23	MNP109	Hipposideridae	<i>Hipposideros galeritus</i>	F	7.0	45.3	30.4	10.0	N/A	5.3
Jun-23	MNP110	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	11.0	47.9	N/A	18.5	N/A	7.0
Jun-23	MNP111	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	8.0	40.3	16.9	12.5	N/A	5.5
Jun-23	MNP112	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	47.5	N/A	15.3	N/A	7.4

Table 2 continued

Jun-23	MNP113	Rhinolophidae	<i>Rhinolophus borneensis</i>	M	7.0	40.1	20.4	14.4	N/A	5.4
Jun-23	MNP114	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	12.0	50.4	24.7	23.2	N/A	6.2
Jun-23	MNP115	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	50.0	N/A	14.6	N/A	8.5
Jun-23	MNP116	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	48.5	N/A	12.5	N/A	7.8
Jun-23	MNP117	Rhinolophidae	<i>Rhinolophus philippinensis</i>		11.0	51.1	22.5	25.3	N/A	6.0
Jun-23	MNP118	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	46.8	N/A	15.3	N/A	7.2
Jun-23	MNP119	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	47.8	N/A	17.7	N/A	7.1

Table 2 continued

Jun-23	MNP120	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	9.0	46.8	N/A	16.4	N/A	7.5
Jun-23	MNP121	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	46.1	N/A	18.4	N/A	7.1
Jun-23	MNP122	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	11.0	46.4	N/A	18.0	N/A	7.0
Jun-23	MNP123	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	48.6	N/A	16.5	N/A	7.5
Jun-23	MNP124	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	47.2	N/A	16.3	N/A	7.1
Jun-23	MNP125	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	47.9	N/A	16.7	N/A	7.2
Jun-23	MNP126	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	7.0	41.4	N/A	15.3	N/A	6.9

Table 2 continued

Jun-23	MNP127	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	11.0	50.6	N/A	27.0	N/A	6.3
Jun-23	MNP128	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	10.0	41.3	N/A	23.0	N/A	6.7
Jun-23	MNP129	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	10.0	50.0	N/A	17.7	N/A	7.9
Jun-23	MNP130	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	47.1	N/A	17.2	N/A	7.1
Jun-23	MNP131	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	47.3	N/A	17.3	N/A	7.0
Jun-23	MNP132	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	47.9	N/A	16.6	N/A	6.8
Jun-23	MNP133	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	10.0	51.1	N/A	26.3	N/A	6.7

Table 2 continued

Jun-23	MNP134	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	48.3	N/A	16.0	N/A	6.9
Jun-23	MNP135	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	11.0	47.5	N/A	15.0	N/A	7.6
Jun-23	MNP136	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	13.0	47.2	N/A	17.8	N/A	6.8
Jun-23	MNP137	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	8.0	47.6	N/A	17.3	N/A	7.5
Jun-23	MNP138	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	12.0	36.2	N/A	17.4	N/A	6.9
Jun-23	MNP139	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	47.7	N/A	17.6	N/A	7.6
Jun-23	MNP140	Rhinolophidae	<i>Rhinolophus borneensis</i>	F	11.0	33.0	N/A	15.2	N/A	5.5

Table 2 continued

Jun-23	MNP141	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	48.8	N/A	17.0	N/A	7.8
Jun-23	MNP142	Rhinolophidae	<i>Rhinolophus creaghi</i>	M	9.0	48.6	N/A	17.1	N/A	7.5
Jun-23	MNP143	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	12.0	45.3	N/A	16.8	N/A	6.4
Jun-23	MNP144	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	49.2	N/A	16.8	N/A	7.0
Jun-23	MNP145	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	11.0	50.0	26.2	24.3	N/A	6.9
Jun-23	MNP146	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	48.1	N/A	17.6	N/A	6.5
Jun-23	MNP147	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	9.0	37.6	N/A	17.7	N/A	7.4

Table 2 continued

Jun-23	MNP148	Rhinolophidae	<i>Rhinolophus creaghi</i>	F	10.0	48.1	N/A	17.7	N/A	7.0
Jun-23	MNP149	Hipposideridae	<i>Hipposideros galeritus</i>	F	9.0	47.4	31.3	11.5	N/A	4.5
Jun-23	MNP150	Hipposideridae	<i>Hipposideros galeritus</i>	M	8.0	45.8	22.9	12.3	N/A	4.7
Jun-23	MNP151	Rhinolophidae	<i>Rhinolophus philippinensis</i>	F	12.0	51.9	24.6	27.1	N/A	6.6
Jun-23	MNP152	Hipposideridae	<i>Hipposideros galeritus</i>	M	6.0	45.9	31.5	12.6	N/A	4.3
Jun-23	MNP153	Rhinolophidae	<i>Rhinolophus philippinensis</i>	M	11.0	51.3	25.7	28.7	N/A	6.5
Jun-23	MNP154	Hipposideridae	<i>Hipposideros galeritus</i>	M	7.0	45.6	31.1	10.5	N/A	4.3

Appendix F: Detection of BtCoV-positive sequences using Q-assay

Table 3: BtCoV positive sequences using Q-assay in this study.

Accession ID	Sequence ID	Host-Family	Host-Species	Virus-Genus	Virus-Subgenus	Lineage	Region
LC858043	NNP064q	Miniopteridae	<i>Miniopterus magnater</i>	Alphacoronavirus	<i>Minunacovirus-related coronavirus</i>	Clade A1	Northern: Niah National Park
LC855489	MNP151q	Rhinolophidae	<i>Rhinolophus philippinensis</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern: Mulu National Park
LC858047	MNP077q	Rhinolophidae	<i>Rhinolophus borneensis</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern: Mulu National Park
LC858046	MNP018q	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern: Mulu National Park
LC858048	MNP127q	Rhinolophidae	<i>Rhinolophus philippinensis</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern: Mulu National Park

Table 3 continued

LC868020	MNP023q	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern:Mulu National Park
LC858050	MNP057q	Hipposideridae	<i>Hipposideros cervinus</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern:Mulu National Park
LC858051	MNP063q	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern:Mulu National Park
LC858049	MNP002q	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A2	Northern:Mulu National Park
LC858052	KPP5004q	Pteropodidae	<i>Eonycteris spelaea</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC858053	KPG008q	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC858059	MG066q	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western

Table 3 continued

LC858056	MG052q	Pteropodidae	<i>Macroglossus minimus</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC858058	MG065q	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC858055	MG023q	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC858057	MG055q	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	Nobecovirus-related coronavirus	Clade B1	Western
LC858054	KBP1002q	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	Nobecovirus-related coronavirus	Clade B1	Western
LC858061	MNP138q	Rhinolophidae	<i>Rhinolophus creaghi</i>	Betacoronavirus	Nobecovirus-related coronavirus	Clade B1	Northern:Mulu National Park
LC858060	MNP105q	Hipposideridae	<i>Hipposideros galeritus</i>	Betacoronavirus	Nobecovirus-related coronavirus	Clade B1	Northern:Mulu National Park

Table 3 continued

LC858062	KBP5008q	Pteropodidae	<i>Penthetor lucasi</i>	Betacoronavirus	Nobecovirus-related coronavirus	Clade B1	Western
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Appendix G: Detection of BtCoV-positive sequences using W-assay

Table 4: BtCoV positive sequences using W-assay in this study.

Accession ID	Sequence ID	Host-Family	Host-Species	Virus-Genus	Virus-Subgenus	Lineage	Region
LC863021	NNP064w	Miniopteridae	<i>Miniopterus magnater</i>	Alphacoronavirus	<i>Minunacovirus-related coronavirus</i>	Clade A1	Northern: Niah National Park
LC863026	KBP3001w	Pteropodidae	<i>Dycopterus spadiceus</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A3	Western
LC863024	MNP137w	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A3	Northern:Mulu National Park
LC863028	KBP5012w	Rhinolophidae	<i>Rhinolophus luctus</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A3	Western
LC863027	KBP3005w	Pteropodidae	<i>Cynopterus brachyotis</i>	Alphacoronavirus	<i>Unclassified Borneo-Alphacoronavirus</i>	Clade A3	Western

Table 4 continued

LC863025	MG036w	Pteropodidae	<i>Cynopterus brachyotis</i>	Alphacoronavirus	<i>Unclassified Borneo- Alphacoronavirus</i>	Clade A3	Western
LC863022	MNP002w	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Rhinovirus-related coronavirus</i>	Clade A4	Northern:Mulu National Park
LC863023	MNP023w	Rhinolophidae	<i>Rhinolophus creaghi</i>	Alphacoronavirus	<i>Rhinovirus-related coronavirus</i>	Clade A4	Northern:Mulu National Park
LC863039	MNP063w	Rhinolophidae	<i>Rhinolophus creaghi</i>	Betacoronavirus	<i>Sarbecovirus-related coronavirus</i>	Clade B2	Northern:Mulu National Park
LC863038	MNP050w	Hipposideridae	<i>Hipposideros cervinus</i>	Betacoronavirus	<i>Sarbecovirus-related coronavirus</i>	Clade B2	Northern:Mulu National Park
LC863037	MG066w	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863036	MG065w	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western

Table 4 continued

LC863035	MG055w	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863033	MG023w	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863030	KPG008w	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863032	KPP4009w	Pteropodidae	<i>Penthetor lucasi</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863029	KBP1002w	Pteropodidae	<i>Cynopterus brachyotis</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863031	KPG021w	Pteropodidae	<i>Penthetor lucasi</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western
LC863034	MG045w	Pteropodidae	<i>Penthetor lucasi</i>	Betacoronavirus	<i>Nobecovirus-related coronavirus</i>	Clade B1	Western

Appendix H: Reference RdRp sequences from GenBank used in Q-assay phylogenetic tree.

Table 5: Reference coronavirus RdRp gene sequences from GenBank used in the phylogenetic analysis of the Q-Assay

CoV Genera	Subgenera	Abbreviation	Full name	Host	Collection Date	Country	GenBank Accession Number
α	<i>Tegacovirus</i>	TGEV	Transmissible gastroenteritis virus	Porcine	1952	USA	DQ811789
		HuPn-2018	Canine coronavirus-human pneumonia-2018	Human	2017	Malaysia	MW591993
	<i>Setracovirus</i>	HCoV-NL63	Human coronavirus NL63	Human	2003	Netherlands	NC005831
	<i>Duvinacovirus</i>	HCoV-229E	Human coronavirus 229E	Human	1966	USA	NC002645

Table 5 continued

	<i>Minunacovirus-rCoVs</i>	MpGD17	Bat Coronavirus MpGD17	Bat	2017	China	OQ175069
		1B-AFCD307	Bat coronavirus 1B strain AFCD307	Bat	2006	Hong Kong	EU420137
	<i>Pedacovirus</i>	PEDV CV777	Porcine epidemic diarrhea virus	Porcine	1978	UK	AF353511
	<i>Unclassified Alpha CoV</i>	JOAB0995	Coronavirus PREDICT_CoV-65	Bat	2018	Jordan	MT063888
		HpYN13	Alphacoronavirus sp. strain bat/Yunnan/HpYN13/2019	Bat	2019	China	MZ081390
		PSW01930	Coronavirus PREDICT_CoV-52	Bat	2016	Malaysia	MT064663

Table 5 continued

		PSW01471	Coronavirus PREDICT_CoV-78	Bat	2015	Malaysia	MT064634
β	<i>Merbecovirus</i>	MERS-CoV	Human betacoronavirus 2c EMC/2012	Human	2012	KSA	JX869059
	<i>Embecovirus</i>	HCoV-OC43	Human coronavirus OC43	Human	1967	UK	AY391777
		HCoV-HKU1	Human coronavirus HKU1	Human	2004	China	AY597011
	<i>Sarbecovirus</i>	Bat-SL-CoVZC45	Bat SARS-like coronavirus	Bat	2017	China	MG772933

Table 5 continued

		Rh-BatCoV- RaTG13	<i>Rhinolophus</i> bat coronavirus RaTG13	Bat	2013	China	MN996532
		SARS-CoV-2	Wuhan novel coronavirus	Human	2019	China	MN908947
		BANAL-20-52	Bat coronavirus	Bat	2020	Laos	MZ937000
	<i>Nobecovirus</i>	HKU9-4	Bat coronavirus HKU9-4	Bat	2007	China	EF065516
		Diliman1525G2	Bat coronavirus Philippines/Diliman1525G2/2008	Bat	2008	Indonesia	MT064820
		PSW02710	Coronavirus PREDICT_CoV-76	Bat	2019	Malaysia	MT064806

Table 5 continued

		THAB18358	Coronavirus PREDICT_CoV-17	Bat	2018	Thailand	MT064984
		GCCDC1 356	Rousettus bat coronavirus	Bat	2014	China	KU762338

Appendix I: Reference RdRp sequences from GenBank used in W-assay phylogenetic tree.

Table 6: Reference coronavirus RdRp gene sequences from GenBank used in the phylogenetic analysis of the W-Assay

CoV Genera	Subgenera	Abbreviation	Full name	Host	Collection Date	Country	GenBank Accession Number
α	<i>Tegacovirus</i>	TGEV	Transmissible gastroenteritis virus	Porcine	1952	USA	DQ811789
		HuPn-2018	Canine coronavirus- human pneumonia-2018	Human	2017	Malaysia	MW591993
	<i>Setracovirus</i>	HCoV-NL63	Human coronavirus NL63	Human	2003	Netherlands	NC005831
	<i>Duvinacovirus</i>	HCoV-229E	Human coronavirus 229E	Human	1966	USA	NC002645

Table 6 continued

	<i>Minunacovirus-rCoVs</i>	HKU8	Bat coronavirus HKU8 strain AFCD77	Bat	2006	Hong Kong	EU420139
	<i>Pedacovirus</i>	PEDV CV777	Porcine epidemic diarrhea virus	Porcine	1978	UK	AF353511
	<i>Unclassified Alpha CoV</i>	PSW00557	Coronavirus PREDICT_CoV-52	Bat	2013	Malaysia	KX284940
		PSW01525	Coronavirus PREDICT_CoV-52	Bat	2015	Malaysia	MT083283
	<i>Rhinocovirus-rCoVs</i>	SADS-rCoV	Swine acute diarrhea syndrome related coronavirus	Bat	2014	China	MF769476

Table 6 continued

		HKU2	Rhinolophus bat coronavirus HKU2	Bat	2016	China	MN312347
β	<i>Merbecovirus</i>	MERS-CoV	Human betacoronavirus 2c EMC/2012	Human	2012	KSA	JX869059
	<i>Embecovirus</i>	HCoV-OC43	Human coronavirus OC43	Human	1967	UK	AY391777
		HCoV-HKU1	Human coronavirus HKU1	Human	2004	China	AY597011
	<i>Sarbecovirus</i>	SARS-CoV-1	SARS coronavirus Tor2	Human	2003	Canada	AY274119
		SARS-CoV-2	Wuhan novel coronavirus	Human	2019	China	MN908947

Table 6 continued

		Rh-BatCoV- RaTG13	<i>Rhinolophus</i> bat coronavirus RaTG13	Bat	2013	China	MN996532
		BANAL-20-52	Bat coronavirus	Bat	2020	Laos	MZ937000
		Bat-SL- CoVZC45	Bat SARS-like coronavirus	Bat	2017	China	MG772933
		SZ140288	Bat SARS-like coronavirus	Bat	N/A	China	MN312858
		PSW01983	Coronavirus PREDICT_CoV-84	Bat	2016	Malaysia	MT083289

Table 6 continued

		PSW00163	Coronavirus PREDICT_CoV-51	Bat	2012	Malaysia	KX284939
	<i>Nobecovirus</i>	KAN15_FF508	Bat coronavirus	Bat	2015	Cambodia	MW507215
		B55700-1	Bat coronavirus	Bat	2012	Thailand	KJ868721
		MCL-19-Bat- 748	Coronavirus PREDICT_CoV-17	Bat	2019	India	MT350391
		THAB18414	Coronavirus PREDICT_CoV-17	Bat	2018	Thailand	MT083680
		GCCDC1 356	Rousettus bat coronavirus	Bat	2014	China	KU762338