



Faculty of Sciences

Department of Biological Sciences and Biotechnology

**MOLECULAR-BASED DETECTION OF
CORONAVIRIDAE AND PARAMYXOVIRIDAE IN FECAL
SAMPLES OF BATS INHABITING THE GCWIHABA
CAVES, NORTHWESTERN BOTSWANA**

by

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List of abbreviations

aa	amino acid
AlphaCoV	Alphacoronavirus
BetaCoV	Betacoronavirus
BSC	Biosafety cabinet
CoV	Coronavirus
DeltaCoV	Deltacoronavirus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EIDs	Emerging infectious diseases
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
HCoV	Human coronavirus
MERS-CoV	Middle Eastern Respiratory Syndrome Coronavirus
n.d	no date
nt	nucleotide
PCR	Polymerase Chain Reaction
PIV	Parainfluenza virus
PMV	Paramyxovirus
PPRs	Pattern Recognition Receptors
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RTC	Replicase-transcriptase complex
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe acute respiratory syndrome
SeV	Sendai virus
ssRNA	Single stranded RNA
TAE	Tris-acetate ethylenediaminetetraacetic acid
TLRs	Toll-like receptors
TRS	Transcriptional regulatory sequences

TRS-B	Transcriptional regulatory sequences-bodies
TRS-L	Leader transcriptional regulatory sequences
UTR	Untranslated regions
VLP	Viral-like particles
VSV	Vesicular stomatis virus

Declaration and copyright

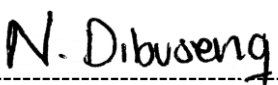
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Abstract

Bats have been associated with emerging and re-emerging viral diseases of public health concern, including the most recent pandemic, COVID-19, caused by a coronavirus named **Severe acute respiratory syndrome coronavirus 2** (SARS-CoV-2). A number of zoonotic viruses have been detected in bats globally, including countries bordering Botswana, such as South Africa, Zimbabwe, and Zambia. Given the potential public health risk of these viruses, the current study sought to detect the presence of members of the *Coronaviridae* (CoV) and *Paramyxoviridae* (**PMV**) in bats fecal samples. A total of 173 fecal samples we collected non-invasively from insectivorous bats that inhabit the Gcwihaba caves. Detection was done using heminested-RT-PCR to determine the presence of viral RNA from the two virus families in the fecal samples. From the sample batches assessed, 2.31% (4/173) and 30.41% (52/173) tested positive for CoV and **PMV** RNA, respectively. On further assessment of sequences with phylogenetic analysis and pair-wise identity, the CoV positives were identified to belong to the *Betacoronavirus* (subgenus: *Merbecovirus*), and **PMV** positives belonged to the *Jeilongvirus*-related viruses, with some being putative jeilongviruses. These findings, provide vital information on the genus of bat-associated viruses in Botswana, specifically the viruses in circulation within the Gcwihaba cave bats. The detection of these viruses and their excretion into the environment has a possible public implication, as the presence of viruses within the bat population of Gcwihaba may pose a health risk to those who visit the caves for tourism. Furthermore, there is a possibility that related viruses may be in circulation in other bats of Botswana, warranting the necessity for further surveillance of these viruses within the country, particularly where the human-bat interaction interface is higher, such as public buildings and homes.

Chapter 1: Introduction

Bats belong to the order **Chiroptera** in the class Mammalia; and are second to rodents in terms of diversity (Smith et al., 2013). They occupy a wide range of geographical niches except extreme climates such as deserts and **polar regions**. Their habitats include caves, tree/thick vegetation canopies, as well as human habitation such as ceilings of houses or public buildings (Irving et al., 2021). In Botswana, one such habitat occupied by bats is the Gcwihaba cave systems, located in the Ngamiland district in Northwest of the country, 20°01' 23"S, 21°21' 19"E (**Figure 1.1**).

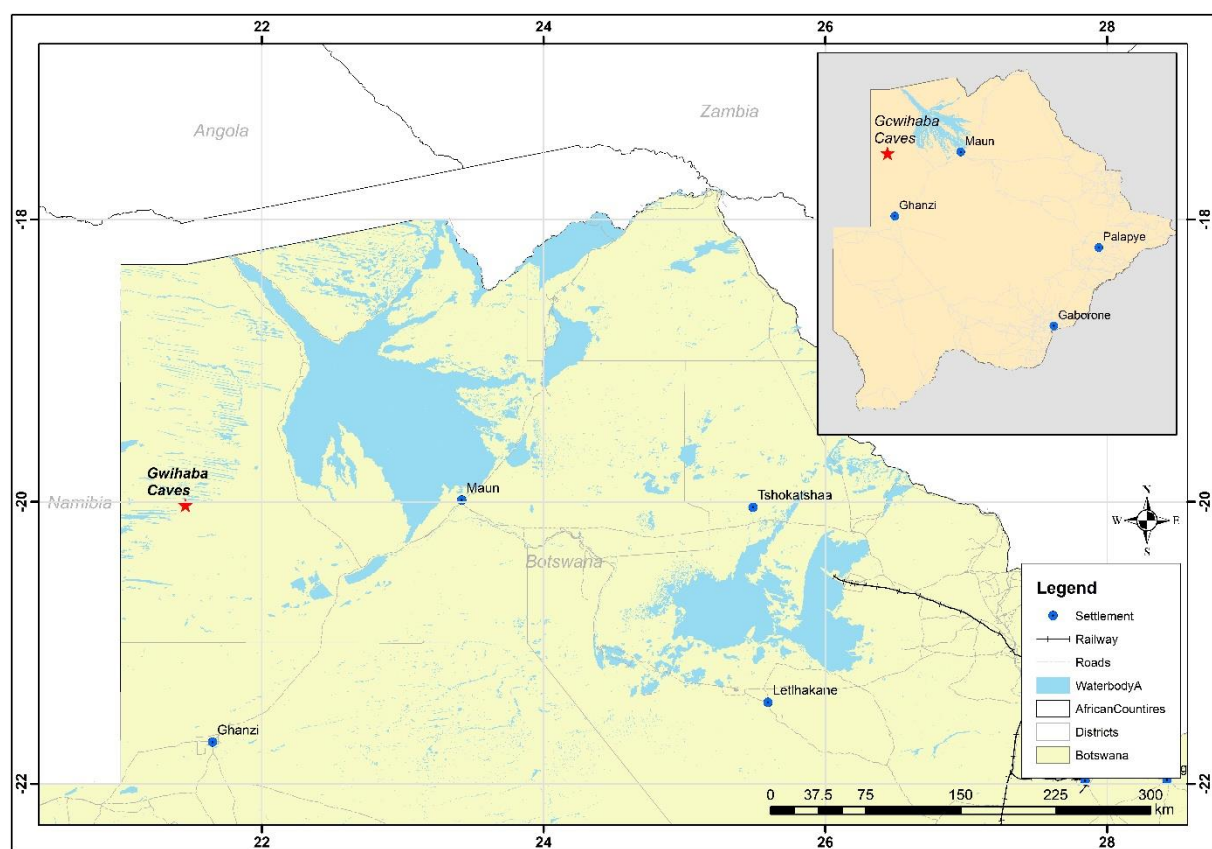


Figure 1.1: Location of the Gcwihaba caves in Botswana

The Gcwihaba caves are protected under the Botswana Monuments and Relics Act (CAP.59.03 of 1970) and were submitted for inclusion in the **United Nations Educational, Scientific and Cultural Organization** (UNESCO) heritage site in 2010 (UNESCO World Heritage Convention, n.d.). The cave was previously home to three San communities: the Gana, Gwiwi and the Gakhwe people. There is evidence of habitation by these communities due to the animal paintings that can be found on some of the cave walls. Currently, the caves

are home to different animal species including porcupines and bats, and remains of unidentified animals have also been found within the cave (Botswana Tourism Organisation, n.d.). Though not listed under tourist attractions in Botswana, the caves are an attraction to cave enthusiasts and bat watchers (Kasso & Balakrishnan, 2013; Mbaiwa & Sakuze, 2009). Additionally, the bats likely provide pest control by feeding on insects that affect agricultural crops in the farming communities that reside around the cave system (Medellin et al., 2017). The cave system consists of a series of mini channels that feed into the main chamber known as Drotsky's chamber. The main composition of the caves is secondary flowstone and dripstone formations (see **Figure 1.2**) (Cooke & Baillieul, 1974). Very few studies have been carried out on the diversity of life forms in the caves. One study by Ferguson et al., (2010) gives an overview of some of the mammals that have been reported in and around the cave, including shrews and rodents such as porcupines, rats and mice. Another study was conducted by Mazebedi and Hesselberg (2020) in which they described the diversity of invertebrates. The latest, on fungal diversity, was carried out by the Nkwe research group, resulting in the discovery and description of a new *Aspergillus* species (Visagie et al., 2021). In addition, the report compiled by Pete Kenyon as part of the Speleological Exploration Club Bulletin, indicated that the caves are inhabited by three distinct bat species, the striped leaf-nosed bat (*Macronycteris vittatus*), Common slit-faces bat (*Nycteris thebaica*) and horseshoe bats (*Rhinolophus* spp.) (Redmayne-Smith, 2015). Bats are a key species in maintaining the ecosystem of the Gcwihaba caves. This can be seen in cave dwelling bats of Cambodia, as reported by Sothearen et al. (2014), indicating that the excretory material (guano) carries nutrients (phosphates and nitrogen) that help support other organisms that only reside in the cave. This is particularly important as some organisms that survive within the cave do not exit the caves, thus the guano becomes their primary source of nutrients (Vanderwolf et al., 2013).

Bats host a wide diversity of microbial agents (Dimkić et al., 2021; Karunarathna et al., 2023; Mühldorfer, 2013), including viruses. Although bats usually host a variety of viruses that cause them little to no harm, some of these viruses (e.g. paramyxoviruses) may cause diseases when transmitted to other hosts (Allocati et al., 2016; Mougari et al., 2022). Other viral pathogens, such as coronaviruses, severe acute respiratory syndrome coronavirus (SARS-CoV) and **SARS-CoV-2**, have had economic implications globally. For example, the COVID-19 pandemic, caused by the coronavirus, **SARS-CoV-2**, had a projected ~\$2 trillion loss in worldwide income (UNCTAD, 2020). **Notably**, the root cause of outbreaks/pandemics is usually a consequence of human activities in disturbing bats and **their** natural environment (Beena & Saikumar, 2019; Wang & Anderson, 2019).

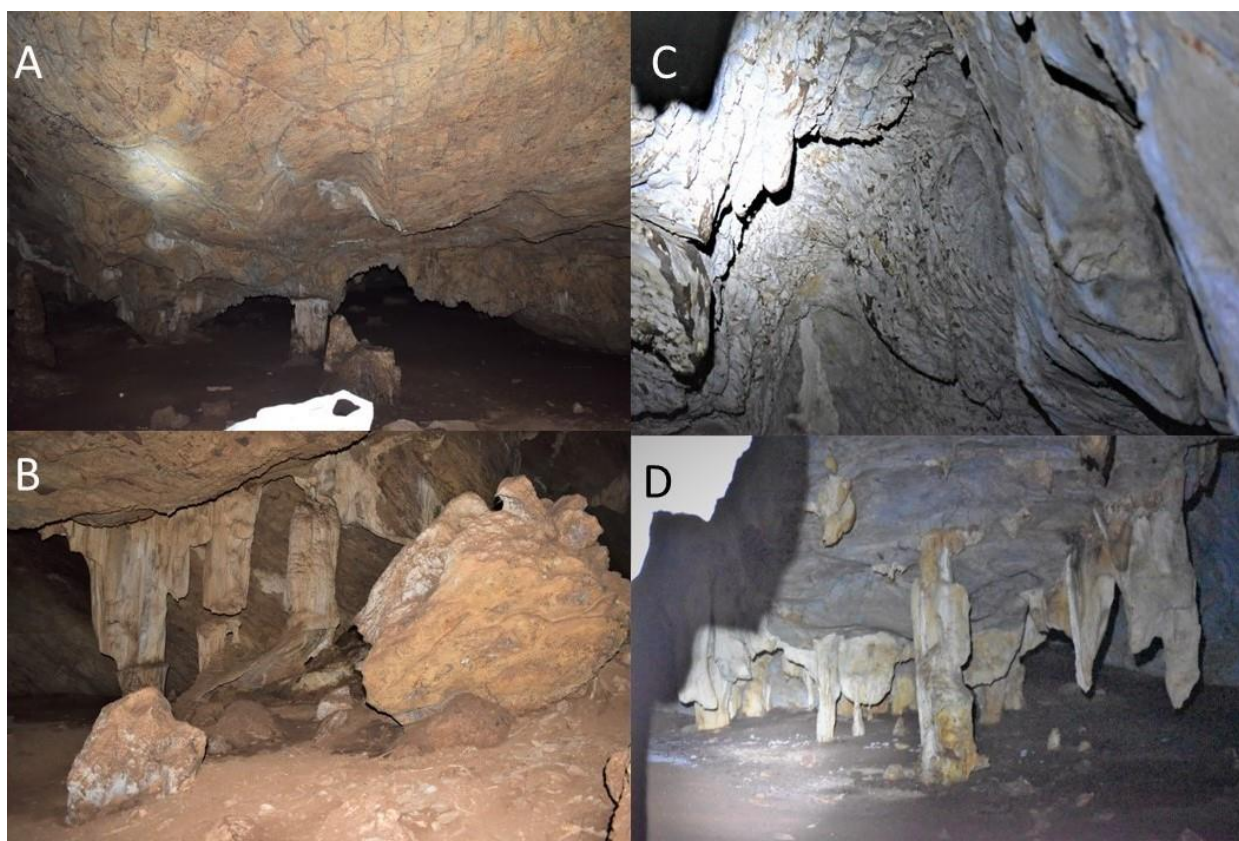


Figure 1.2: Gcwihaba cave rock formations

Low hanging stalactites and stalagmites were found close to the second cave entrance (**A&B**). Stalactites formations are as a result of water and minerals continuously dripping from the ceiling. Stalagmites are also mineral deposits that are formed by water dripping to the cave floor. **C** Limestone crevice that forms a portion of the roof in one of the cave passages. **D**, Chalk-colored low hanging stalactites observed in one of the small chambers further from the cave entrance. Some of these formations run from the parent rock and to the cave floor.

This chapter gives an overview of zoonotic pathogens, and bats as natural hosts of certain virus pathogens. Background on bat-virus dynamics is explained, and additionally, characteristics of bats that have made them successful natural hosts of viral pathogens is reviewed. Emphasis was primarily on the two families *Coronaviridae* and *Paramyxoviridae* within the Sub-Saharan region. The biology of the respective families i.e., taxonomy, genomic and virion structure function, replication, and pathogenesis are described in detail. **Finally**, this chapter gives justification for this study and outlines its aim and objectives.

1.1 Cave-dwelling bats and virus dynamics

Caves are capable of supporting animal and microbial life despite being characterized as inhospitable environments (Mammola, 2019). The notable characteristics of cave systems include the absence of direct sunlight and varying levels of temperature, airflow, and humidity within different sections of the cave (Biagioli et al., 2023; Gabriel & Northup, 2013; Howarth & Moldovan, 2018). These characteristics have provided a stable habitat for bats, and it is also

hypothesized that they offer optimal conditions for microorganisms to thrive, such as viruses, within and outside the bat host (Chan et al., 2011; Fogarty et al., 2008; Willoughby et al., 2017). Caves offer a wide range of structures (e.g. chambers, crevices, and passages) where diverse bat hosts roost (Willoughby et al., 2017). Other inhabitants of caves have adapted so that they can thrive within such an environment, being, directly and indirectly, dependent on bat guano as a nutritional substrate source, a heat source through its fermentation, and a mineral source through undergoing numerous reactions (Kasso & Balakrishnan, 2013; Sakoui et al., 2020).

The complex study of co-evolution and virus sharing in cave-dwelling bats includes consideration of various factors. However, the contribution of genetic adaptation of the host's immune system is essential in the co-evolution of viruses alongside bats. As discussed in section 1.3, bats have evolved alongside viruses, with adaptation of their immune response being one of the main elements that has enabled them to become suitable viral reservoirs. The co-evolution of some viral strains is illustrated in various studies showing an association between SARS-CoV-related viruses and horseshoe bats (*Rhinolophus spp.*) (Temmam et al., 2022).

Concerning viral sharing, many cave-dwelling bats reportedly live in dense colonies, which is thought to facilitate viral transmission through direct contact (Willoughby et al., 2017). However, it is important to note that not all cave-dwelling bats exhibit dense colony behavior. For example, greater horseshoe bats (*Rhinolophus ferrumequinum*) tend to roost singly or as a small colony. Ruiz-Aravena et al. (2022), explain how colony composition, size and density play a role in virus prevalence within a cave system. Ruiz-Aravena et al. noted that multiple bat species that roost closely together in large, dense colonies promote high viral prevalence and sharing. These findings were further supported by Willoughby et al. (2017), who reported viral sharing in similar colony size and density within a mixed roost colony. The bat composition and density results in close contact between individuals of different species (interspecific contact), who would typically have low interactions. Furthermore, the reproductive behavior (synchronized and seasonal breeding) of bats is also a factor in viral sharing within a single species (intraspecific transmission), such as viral transmission within maternity colonies where synchronized and seasonal breeding introduce susceptible individuals (Cappelle et al., 2021; Drexler et al., 2011; Willoughby et al., 2017).

The combined consequences of co-evolution and viral sharing dynamics within bats has resulted in a certain degree of host specificity (Liang et al., 2021). Some viruses have been detected in bats that exhibit distant phylogenetic relatedness, while in other instances, a virus

evolves within a specific bat species (Cui et al., 2007; Willoughby et al., 2017). In the case of distantly related bats sharing viruses, interspecific interactions (contact between different species) have been suggested to promote viral host switching, i.e., when a virus extends host range from one single host species. This is demonstrated by the findings by Lau et al. (2012), where paramyxoviruses were found to be shared by *Miniopterus griveaudi* (family Miniopteridae) and *Myotis goudoti* (family Vespertilionidae). Other instances of possible viral sharing promoted by interspecies contact is reported in a study by Mélade et al. (2016) where AlphaCoV sharing occurred between *Rousettus leschenaulti* and *Hipposideros pomona*. Reports have demonstrated that certain strains of SARS coronaviruses are associated with horseshoe bats, indicating a selection of these hosts by the viruses (Lau et al., 2005; Temmam et al., 2022; Woo et al., 2006). Additionally, Maganga et al. (2020) shows the transmission of coronaviruses between *Hipposideros cf. ruber* and *H. gigas*, despite the two species occupying distinct areas within the cave. Phylogeny of both virus and host, geographical location of the bat host, and physiological and behavioral traits of bats play a significant role in viral sharing, evolution, and specificity (Albery et al., 2020; Liang et al., 2021; McKee et al., 2019; Poulin et al., 2011). Therefore, the Gcwihaba caves emerge as a unique opportunity for investigating bat-virus interactions in Botswana. These caves stand out as ecologically rich and geographically distinct, but also serve as habitat for various bat species.

1.2 Zoonotic pathogens

Zoonotic pathogens (including bacterial, fungal, and viral agents) are ubiquitous and have been reported from a wide range of animal hosts, such as birds, wildlife and domestic animals (Morse et al., 2012; Ramanantsalama et al., 2022). In some instances, zoonotic pathogens have been associated with contaminated food and water sources (Rahman et al., 2020). These pathogens, known as zoonotic, cross species barrier, causing a wide range of diseases. Therefore, they have been at the forefront of emerging infectious diseases (EIDs), especially of viral nature. Disease outbreaks such as the 1918 Spanish flu led to ~50 million deaths, whilst the Hong Kong flu led to ~700 000 deaths (Bean et al., 2013). Recent emerging and re-emerging zoonotic diseases that have been of public health concern include the following viral diseases: Ebola, detected in Uganda in late 2022 (Samarasekera, 2023); Severe acute respiratory syndrome (SARS) was reported worldwide; Hendra virus in Australia in 2010 (Playford et al., 2010); Nipah virus in India in 2018 (Yadav et al., 2022); and Middle East respiratory syndrome (MERS) in the middle east in 2012 (Mubarak et al., 2019), as well as South Korea in 2015 (Kim et al., 2017). A summary of bats and some of their associated viral pathogens are shown in Table 1. The resulting outbreaks have had a significant impact on

infected animal and human populations. Disease outbreaks may also have detrimental effects on the agricultural sector at household or national levels, particularly those who keep livestock for sale and consumption, as it would require the culling of the entire flock, whether infected or not (Smith & Wang, 2013).

Table 1: Bats species and their associated viral pathogens

Bat species	Associated Viral Pathogens	Reference
Rousettus bat	Marburg virus, Ebola virus, SARS-like coronaviruses, Lyssaviruses	(Amman et al., 2012; Pawęska et al., 2018; Pourrut et al., 2009; Schuh et al., 2019)
Pipistrelle bats	European bat lyssavirus, Pipistrellus bat coronavirus HKU5	(Lau et al., 2013; Woo et al., 2006, 2007)
Flying fox bats	Tioman virus, Hendra virus, Nipah virus, Australian bat lyssavirus	(Boardman et al., 2020; Drexler et al., 2009; Fogarty et al., 2008; Mackenzie et al., 2001; P. Yadav et al., 2016)
Lesser bamboo bat	Tylonycteris bat coronavirus HKU4	(Lau et al., 2005; W. Li et al., 2005; Woo, Lau, Li, et al., 2012)
Bent-winged bat	Coronaviruses	(D. K.W. Chu et al., 2006; Ge et al., 2016; Poon et al., 2005)
Horseshoe bats	SARS-like coronaviruses, Rhinolophus bat coronaviruses	(Lau et al., 2005, 2007, 2010; W. Li et al., 2005; Poon et al., 2005)
Mouse-eared bats	European bat lyssavirus	(Amengual et al., 2007; Arai et al., 2003)
Roundleaf bats	Bat adeno-associated viruses (AAVs)	(Li et al., 2010)

Transmission of zoonotic pathogen during outbreaks may be through diverse means (as shown in **Figure 1.3**). These include direct contact with host animals, as is seen in the transmission of Ebola which occurs via contact with infected primates (Gumusova et al., 2015); MERS transmission from dromedary camels (Azhar et al., 2014; Mulabbi et al., 2021); and Ebola virus human-human transmission facilitated by traditional burial practices (Pandey. et al., 2014; Manguvo & Mafuvadze, 2015; Markotter et al., 2020; Smith & Wang, 2013). Transmission may also occur through bites from insect vectors such as mosquitoes and ticks. These vectors have been implicated in the transmission of West Nile Virus (Valiakos et al.,

2016), Rift Valley Virus and Tularemia (Akhvlediani et al., 2018). Furthermore, the transmission may also be indirect through close contact with the infected host's habitat usually through fecal-oral transfer, or through contact with the infected animal's fluids. The latter occurs mostly as an occupational hazard for individuals such as veterinaries, slaughterhouses employees, and people involved in the day-to-day care of animals (Epp & Waldner, 2012; Vonesch et al., 2019). Indirect transmission may occur through the consumption of food and water contaminated with zoonotic pathogens such as bacteria [for example, *Salmonella* spp and *Campylobacter* spp. (Abebe et al., 2020)]; or viruses [for example Hepatitis E virus (Yugo & Meng, 2013)].

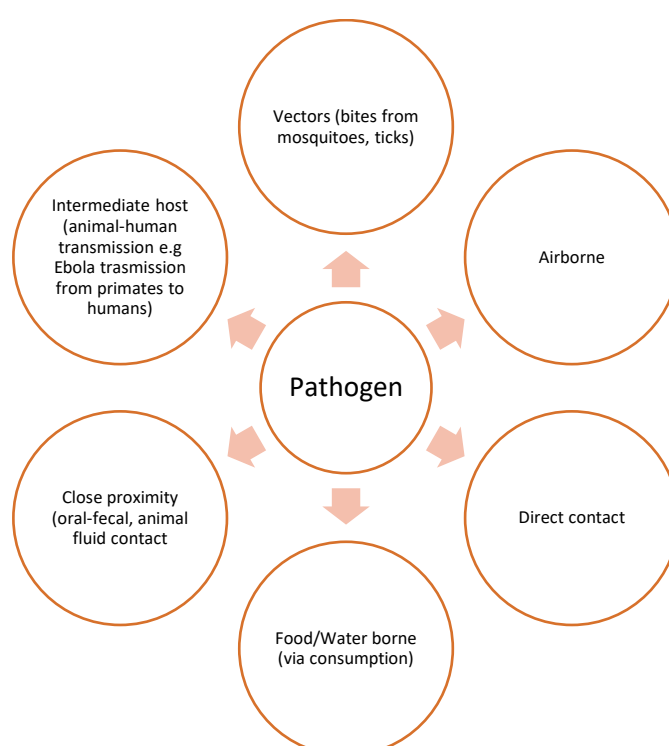


Figure 1.3: Method of transmission of zoonotic pathogens

Certain practices increase the likelihood of the emergence and the spread of zoonotic diseases. Intensive agricultural practices and changes in land use are two of the most common activities that precede cases of increased incidents of new outbreaks of zoonotic diseases (reviewed by Smith & Wang, 2013). Intensive agricultural practices usually involve land clearing (deforestation), which leads to humans encroaching into wildlife habitats, thereby increasing the likelihood of encountering potential pathogens (Beena & Saikumar, 2019; Murray & Daszak, 2013). Also, environmental changes such as climate change may have an impact on the life cycles of vectors that carry zoonotic pathogens. Typically, high rainfall promotes mosquito breeding and life cycle due to large bodies of stagnant water. A positive correlation between mosquito borne zoonotic diseases and high rainfall has been suggested in studies by Sule et al., (2018), and Weaver & Reisen (2010).

The zoonotic agents of interest for this thesis are viral pathogens. Within the last twenty years, viral pathogens have caused global public health unrest. The emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in China, middle eastern respiratory syndrome (MERS) in the Middle East, Ebola virus in Central and West Africa, and lately SARS-CoV-2 in China (Lai et al., 2020; Mubarak et al., 2019; Piot & Spencer, 2018). Although the origin of SARS-CoV2 is still obscure, other viruses mentioned above have been linked to bats as the reservoir, with intermediate hosts that include domestic and wild animals (Hu et al., 2015; Moratelli & Calisher, 2015). Extensive studies carried out have implicated bats and rodents as the **natural hosts** associated with various zoonotic viral pathogens (Luis et al., 2013). **However, recent analyses suggest a more homogeneous distribution of zoonotic risk among taxonomic orders, indicating that bats and rodents may not be unique in this regard (Mollentze & Streicker, 2020)** Therefore, in the current study, I used molecular techniques to investigate the occurrence and diversity of coronaviruses and paramyxoviruses in bats found in the Gcwihaba cave (**Figure 1.1**).

1.3 Bats as natural hosts of viral pathogens

The chiropteran order **comprises** of 21 families with 1470 species (Dutheil et al., 2021; Simmons et al., 2023). Although bats have played key ecological roles such as seed dispersal (Corlett, 2017), pollination (Lim et al., 2018), pest control particularly in vector-borne zoonotic diseases regions (Gonsalves et al., 2013; Kunz et al., 2011), and provide guano that is used as plant manure (Ramírez-Fráncel et al., 2022; Sakoui et al., 2020), they can also be considered as a nuisance. Bats have been implicated as **natural hosts** of potentially deadly pathogens that have previously crossed into other species including humans and upset the global public health systems. Bats can host zoonotic viral pathogens while exhibiting little to no symptoms (Brook & Dobson, 2015). Consequently, they are commonly referred to as natural hosts or reservoirs of viral zoonotic pathogens. **According to recent data, a comprehensive database on bat viruses from 69 countries worldwide comprises more than 4,100 bat-associated animal viruses belonging to 23 viral families, detected in 196 bat species. This highlights the extensive diversity of zoonotic viral pathogens associated with bats** (Beena & Saikumar, 2019). The history of bats and viral zoonoses dates back to the early 20th century when they were associated with the emergence of rabies virus in bats in the 1930s in Brazil and Trinidad, and 1950s in USA (Calisher, 2015; Rupprecht et al., 2022; Kuzmin et al., 2011; Velasco-Villa et al., 2017). In the years that followed, bats were further **suspected to be** associated with the Ebola outbreak in the Zaire region (now known as the Democratic Republic of Congo) in 1976 (Piot & Spencer, 2018); Hendra virus in 1994 in Australia (Bowden et al.,

2001); Nipah virus in Malaysia in 1998/1999 (Wang & Anderson, 2019) as well as in Bangladesh and India in 2001 (Chadha et al., 2006; Luby et al., 2009). Additionally, bats were identified as **natural hosts** for SARS-CoV and MERS-CoV in 2002 and 2012 in China and Saudi Arabia, respectively (Dhaka & Vijay, 2018; Drexler et al., 2014). Various physiological and ecological elements of bats have made bats successful as natural hosts of viruses. These elements have facilitated the successful replication and transmission of viral pathogens within and across bat populations, as well as spillover to their secondary hosts including humans. These attributes are shown in **Figure 1.4** (Brook & Dobson, 2015; Calisher et al., 2006; Van Brussel & Holmes, 2022), and are discussed below in detail.

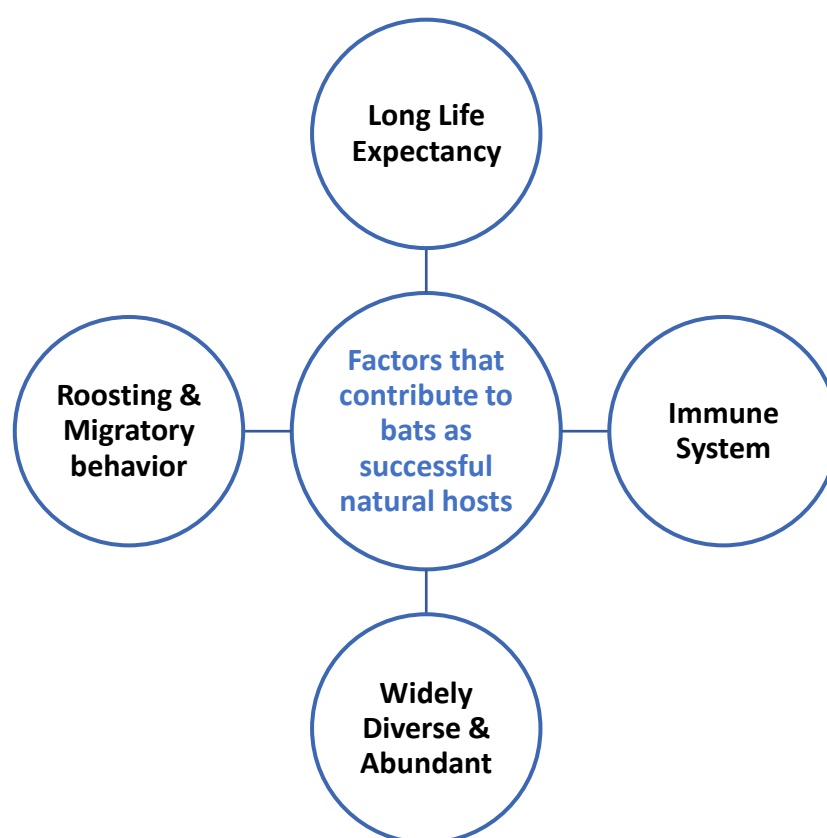


Figure 1.4: Factors that potentially contributes to bats as natural hosts to viral pathogens

1.3.1 Species diversity, abundance and life expectancy

Bats are the second most abundant mammalian species (rodents being the first) (Letko et al., 2020). They account for 25% of the total mammalian species population. There were 1,470 bats species as of July 2023, according to the Bats of the world Taxonomic and Geographic database report (Simmons et al., 2023). Over 1,400 known bat species were traditionally classified into the suborders Megachiroptera (megabats) and Microchiroptera (microbats) (Springer, 2013), with genetic studies later revising the classification to Yangochiroptera

(*Vespertilioniformes*) and Yinpterochiroptera (*Pteropodiformes*) suborders (Amador et al., 2018; Lei & Dong, 2016; Sotero-Caio et al., 2017; Tsagkogeorga et al., 2013). The wide range of species diversity offers an abundant host range where disease-causing zoonotic viral pathogens can successfully replicate and evolve. Various bat species have varying degrees of expected lifespan, but most lead long lives in comparison to similar-sized mammalian species. This longevity is attributed to their roosting behaviour where cave-dwelling bats tend to outlive their open space counterparts, possibly because they have reduced exposure to harsher environmental conditions and predators (Wilkinson & South, 2002). The life expectancy of bats has provided a conducive environment where bat-borne viruses can replicate and evolve. This environment encourages stable virus transmission rates within bat populations (*intra-species transmission*), and from bats to other animals (*inter-species transmission*).

1.3.2 Habitat, roosting and migratory behavior

As bats occupy diverse geographical areas, coupled with their migratory behavior, this enables the introduction or acquisition and dissemination of viral pathogens over a wide geographical range (Calisher et al., 2006; Dhaka & Vijay, 2018; Luis et al., 2013). Bats live in large colonies, especially in their natural habitats. Roosting behaviour in a few instances is associated with more than one species inhabiting a particular habitat. This facilitates the interaction and transmission of viral pathogens among individuals within a species (horizontal transmission), as well as the opportunity to cross the species barrier if the other colony is made of individuals that are susceptible to infection. This is promoted by the proximity among the individuals of a colony or through social interactions such as mating, and grooming (Calisher et al., 2006; Dhaka & Vijay, 2018; Luis et al., 2013).

1.3.3 Innate and adaptive immunity

Our understanding of how bats can host zoonotic viral pathogens while remaining asymptomatic is from single-species studies (Brook & Dobson, 2015; Moratelli & Calisher, 2015; Randolph & Barreiro, 2018). The information generated is then extrapolated to encompass the whole Chiropteran order (Markotter et al., 2020). There are drawbacks to making such extrapolations as the expression of genes that control immune responses varies among host species; what applies to one genus or species may not be applicable to another (Baker et al., 2013; Banerjee et al., 2017). Nonetheless, bat immune factors that make them good natural hosts for viruses are attributed to their pattern recognition receptors (PRRs), complement system activity, and signaling molecules such as cytokines (Banerjee et al., 2020).

PRRs play a vital role in bats upon viral infection. PRRs are the first line of defense for a host upon pathogen infection. This is possible through two mechanisms: detection of endogenous molecules released by cells upon pathogen-related damage (damage-associated molecular pattern molecules); or recognition of conserved molecular structures of the pathogen (pathogen-associated molecular patterns) (Irving et al., 2021). This subsequently activates the host's innate immune response, with adaptive immunity being activated by PRR-inducing signals (Akira et al., 2006; Palm & Medzhitov, 2009). Toll-like receptors (TLRs), one class of PRRs, demonstrate a high conservation pattern within bats and other mammalian hosts (Kumagai & Akira, 2010). Upon viral infection, TLRs identify the viral nucleic acids by pathogen-associated molecular patterns, resulting in the activation of both innate and adaptive immune responses within the host. This involves signaling pathways that result in the production of cytokines and interferons which in turn result in the induction of interferon-stimulated genes (ISGs) which, depending on the virus type, may inhibit or promote viral replication (Schoggins & Rice, 2011).

TLRs such as TLR 3, 7 & 8 (which are homologs to human TLRs 1-10) have been characterized in the frugivorous bat *Pteropus alecto* (Cowled et al., 2011). Other classes of PRRs include retinoic acid-inducible gene I (RIG-I)-like helicases such as RIG-I and melanoma differentiation-associated protein 5 (*mda5*) that have also been characterized within the *P. alecto* species (Cowled et al., 2012). Cytokines of the type-I interferons (IFNs), alpha (α) and beta (β) have been identified in three fruit bats species (*P. vampyrus*, *R. aegyptiacus* and *Dobsonia viridis*) (Zhou et al., 2011, 2016), and they are induced in response to viral pathogen infections. Interestingly, inflammatory cytokines such as tumour necrosis factor alpha (TNF α) are expressed at low levels, a phenomenon contrary to viral infection in human hosts. This allows bat immune systems to detect pathogen infection while minimizing inflammatory responses that would otherwise lead to tissue damage (Banerjee et al., 2017).

For adaptive immunity, both antibody secretion and cell-mediated immunity are key mechanisms in the ability of bats to function as natural hosts. Immunoglobins have been detected in four bat species (*Myotis lucifugus*, *Eptesicus fuscus*, *Cynopterus sphinx* and *Carollia perspicillata*) (Butler et al., 2011). These include isotypes immunoglobins M (IgM), IgE, IgA and IgG, the latter having multiple classes detected (Baker et al., 2010; Wynne et al., 2013). In characterization studies, these immunoglobins are similar to those induced in humans upon infection. Additionally, IgD, known as an ancient isotype, has been detected in *M. lucifugus*, at both transcriptional and genomic levels (Baker et al., 2013; Ohta & Flajnik, 2006). Antibody-specificity is conferred by amino acid residues on the N-terminal of the

variable domain of the heavy and light chains. In addition to antibody specificity, these variations also promote antibody diversity. In some studies, a high antibody repertoire in both megabats and microbats has been observed like that found in humans and mice (Baker et al., 2010).

Studies have been carried out that indicate a variation in the degree of pathogen identification and immune response in bats. A study by Zhang et al., (2013) describes that though many genes are conserved in the mammalian order, there are some which have been lost or their expression significantly reduced. The locus containing the *PYHIN* gene family generally expresses one or more genes within most mammals (Cridland et al., 2012). In studies by Zhang et al. and Ahn et al. (2016), *Myotis davidii* and *P. alecto* (representatives of the two suborders) showed loss of *AIM2* and *IFI16* genes (part of the *PYHIN* locus), which are involved in detection of pathogenic DNA. This could suggest that bats underwent changes in the recognition of microbial DNA recognition. Variation in gene expression extends to Natural killer cell receptors. These include the killer immunoglobulin-like receptors (*KIRs*) and killer cell lectin-like receptors (*KLRs*), which are entirely absent in *P. alecto* and exist as a pseudogene, *Ly49* in *M. davidii* (Papenfuss et al., 2012). The combination of the innate and adaptive immunity features in bats as discussed above, has established bats as good viral pathogen hosts, able to remain asymptomatic or mildly symptomatic.

1.4 Identified bat-borne viral families

As previously indicated, bats are widely able to host diverse viruses presumably asymptotically. A summary of known virus families that have been detected in bats based on sequence data and number of publications identified for each viral family are shown in **Figure 1.5** (Letko et al., 2020). A wide range of DNA and RNA viruses have been identified from different bats using their various biological matter i.e., oral, and rectal swabs, kidney, spleen, etc., though the available information is predominantly sequence-based data. There have been **limited** instances of viral pathogens spilling over to humans, suggesting a relatively low zoonotic potential or exposure events, for example astroviruses. The DNA viruses include members of *Hepadnaviridae* such as **Hepatitis virus** that infects the human liver, *Parvoviridae* which affects both vertebrate and invertebrate hosts (e.g., Parvovirus B19, Human Bocavirus), and *Adenoviridae* which includes viruses that affect a wide range of vertebrate hosts. For example, *Mastadenovirus* affects mammals, *Aviadenovirus* avian species host (Jejesky de Oliveira et al., 2020), *Atadenovirus* affects both avian and reptilian species hosts, *Siadenovirus* affects bird, reptilian and amphibian hosts), and lastly *Ichadenovirus* which affects fish only

(Harrach et al., 2019; Kovács et al., 2010; Rinder et al., 2020; Wachtman & Mansfield, 2012; Wellehan et al., 2004). RNA virus sequences that have been identified from bat samples include members of the *Flaviviridae* such as the West Nile Virus, and *Filoviridae* which are responsible for most of the life-threatening viral hemorrhagic fever including Marburg virus, and Ebola (Ain-Najwa et al., 2020; Emanuel et al., 2018; Jeffrey Root, 2013; Olival & Hayman, 2014). Most sequences that have been identified include members of the *Rhabdoviridae*, *Coronaviridae* and *Paramyxoviridae* (Letko et al., 2020). However, there may be some degree of biasness as several studies were carried out using species-specific or group-specific primers for viral detection. The focus on the three mentioned families may be due to the higher instances of crossover into human populations, severity of health implications following spillover, or outbreak potential. Some viral families may be understudied as a result, when ideally, detection techniques such as viral metagenomics could provide better insight of viral families that exist within host populations (Edwards & Rohwer, 2005). However, such techniques are computationally demanding and expensive, thus would not be ideal for routine surveillance monitoring (Ko et al., 2022). Delwart (2007) has explained that advancement in molecular biology such as pyrosequencing, and availability of molecular grade reagents, as well as advancement in software that allows for greater data analysis through low-level sequence comparison, should drive the quest for understanding viral populations in animals and the environment.

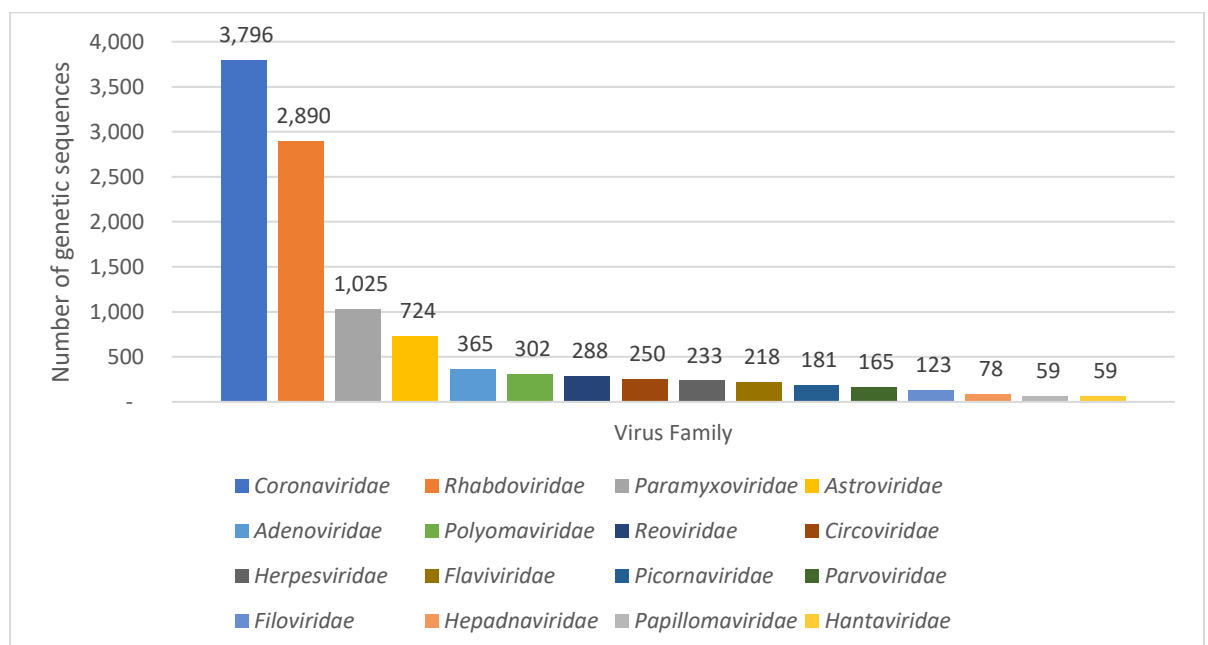


Figure 1.5: Number of viral families detected in bats.

Adapted from Letko et al., (2020).

1.5 Coronaviridae

Coronaviruses are of public health concern and economic importance. High mortality rates in agricultural industries during outbreaks, or the physiological impact on the infected hosts has implications on profits at household or national level. For example, infectious bronchitis virus (IBV) which primarily affects poultry, requires poultry culling, as a disease management technique, thereby affecting trade (Cavanagh, 2007; Colvero et al., 2015). On the human impact of coronaviruses, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2020 has seen **reported cases** at ~7 million globally compared to ~9000 **cases** in the SARS-CoV outbreak in 2002 (Petersen et al., 2020; Shaw, 2006). Surveillance to identify possible zoonotic pathogenic agents of emergence/re-emergence potential are carried out through initiatives such as PREDICT project (Carlson, 2020), the Global Virome Project (GVP) (Carroll et al., 2018), and One Health project (Asokan, 2015). These initiatives have increased the understanding of pathogens such as coronaviruses; more specifically their taxonomy/phylogeny, structure, replication cycle, and their pathogenicity.

1.5.1 Taxonomy

The International committee of taxonomy of viruses (ICTV) (Woo et al., 2023) have classified the *Coronaviridae* family (order *Nidovirales*, suborder *Cornidovirineae*) as depicted in **Figure 1.6** (Corman et al., 2014; Geldenhuys et al., 2018). Three subfamilies are found within the family, and these include *Letovirinae*, *Pitovirinae*, and the widely diverse *Orthoparamyxovirinae* subfamily. The latter includes the genera that have been intensively researched and are currently of public health importance. These include the *Alphacoronavirus* (AlphaCoV), *Betacoronavirus* (BetaCoV), *Deltacoronavirus* (DeltaCoV), and *Gammacoronavirus* (GammaCoV). The AlphaCoVs are further divided into ~14 subgenera, while BetaCoVs are further characterized into five subgenera as indicated in **Figure 1.6** below. Both the AlphaCoVs and BetaCoVs are of clinical importance, with human coronaviruses such as 229E and NL63 being classified under the AlphaCoVs; whilst HKU1, SARS-CoV, and MERS-CoV are classified under the BetaCoVs (Hu et al., 2015). Additionally, similar viruses to MERS-CoV coronaviruses (MERS-like coronaviruses) have been associated and detected in bats during viral surveillance studies globally (Anthony et al., 2017; Chidoti et al., 2022; Drexler et al., 2014; Ithete et al., 2013; Lau et al., 2013; Meta Djomsi et al., 2023; Woo et al., 2006, 2007). The MERS-like coronaviruses are classified under the *Merbecovirus* subgenus within the *Betacoronavirus* subfamily (Wong et al., 2019; Zhou et al., 2021). The main variation within the subgenus is observed within the spike and envelope coding regions (Woo, Lau, Li, et al., 2012).

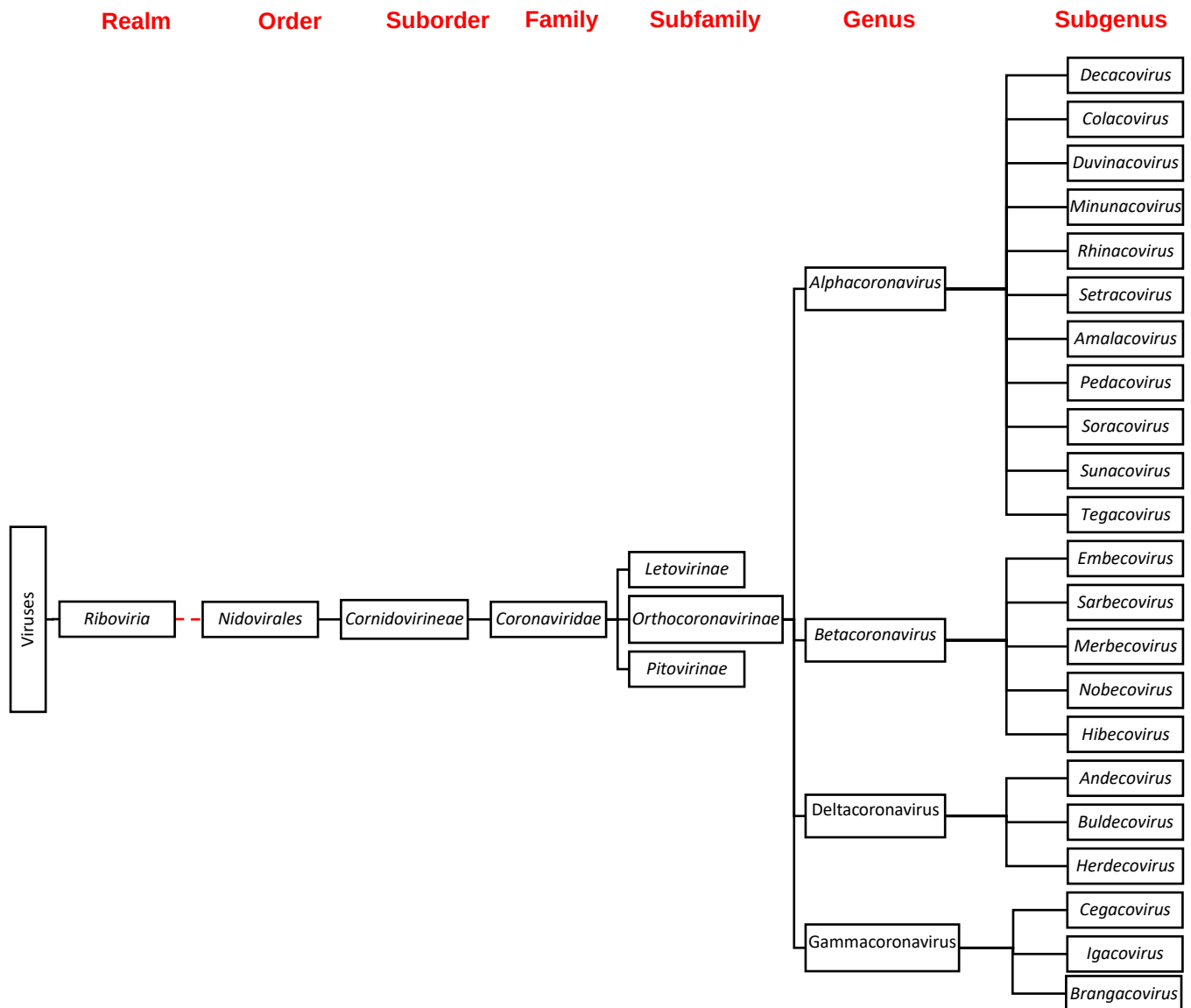


Figure 1.6: *Coronaviridae* taxonomy

1.5.2 Morphology and genomic structure

Members of the *Coronaviridae*, particularly the coronaviruses, are named as such due to their characteristic crown-like structure. This crown-looking structure is due to the club-like structures embedded in the viral membrane and are known as spike proteins (as seen in **Figure 1.7**). Members of this family are enveloped with a non-segmented positive sense ssRNA genome. The ssRNA genome can be directly translated into respective structural and nonstructural proteins in the presence of viral replicase. This is because the ssRNA has a poly-A tail and a methyl cap structure on its 5' end. The overall genome size ranges from 26 kb to 32 kb (Chen et al., 2020; Jonsdottir & Dijkman, 2016; Maier et al., 2015). This large genome structure facilitates extra plasticity in genome modification via mutations or recombination that further promotes interspecies variability, interspecies "host jump" and the

introduction of novel coronaviruses when the conditions are suitable. Genome organization and expression are similar in human and animal coronaviruses.

The genome comprises the open reading frame (ORF) 1a/b on the 5' end and is responsible for the expression of 16 nonstructural proteins (nsp: 1-16). The structural proteins are encoded on the 3' end of the genome, and these include the spike protein (S), envelope (E), membrane (M) and nucleocapsid (N). Variation within the members of the coronavirus subfamily is mainly found in their accessory proteins (Poon et al., 2005).

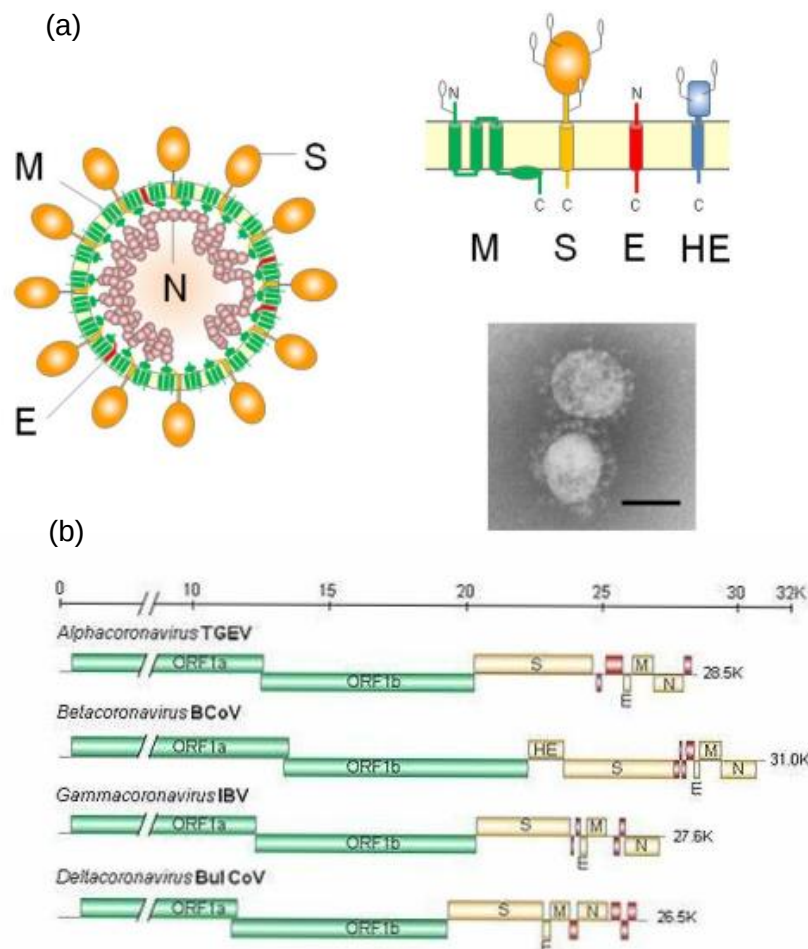


Figure 1.7: Coronavirus morphology and genome structure.

(a) Virion morphology shows the structural proteins including spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. Observation of coronavirus virion observation as viewed under an electron microscope. **(b)** Organization of ~30kb genome. Variation among the Orthocoronavirinae genera (Alpha-, Beta-, Gamma-, and Deltacoronavirus) is also indicated. TGEV- Transmissible gastroenteritis virus; BCoV- Bovine coronavirus; IBV- Infectious bronchitis virus, BulCoV- Bulbul coronavirus HKU11. Taken from Ujike & Taguchi (2015).

1.5.3 Structural proteins and their function

The structural proteins of the coronaviruses are briefly discussed below (**Table 1.2**) as reviewed by (Chen et al., 2020; Fehr & Perlman, 2015; MacLachlan & Dubovi, 2017b; Maier

et al., 2015; Payne, 2020). These proteins play a vital role in viral replication, transcription, viral assembly, and budding process.

Table 1.2: *Coronaviridae* virion protein structure and function

Protein	Size (kDa)	Structure	Functions	Reference
Spike (S)	~150	heavily N-linked glycosylated trimeric protein with two domains, S1 and S2, that act as binding domain and stalk respectively	viral attachment to susceptible host cell receptor; and viral membrane-host cell membrane fusion.	(Fehr & Perlman, 2015)
Membrane (M)	~25-30	exists as a dimer (may alternate between two confirmations)	give virions their overall structure	(Schoeman & Fielding, 2019; Y. Wang et al., 2020)
Envelope (E)	~8-12	integral membrane protein	Facilitates assembly and release	(Schoeman & Fielding, 2019)
Nucleocapsid (N)	~50	two domains: the N-terminal domain (NTD) and the C-terminal domain (CTD). Heavily phosphorylated	binds to the viral RNA genome. essential in overall structural organization. tethers viral genome to the replicase-transcriptase complex (RTC), and subsequent packaging of the nucleocapsidated genome into viral particles.	(Müller et al., 2007)
Hemagglutinin Esterase (HN)	~48	Comprised of three domains: stem, acetylerase domain; and receptor binding domain	binds sialic acids on surface glycoproteins and contains acetyl-esterase activity. may enhance S-protein-mediated cell entry and virus spread	(Payne, 2017a)

1.5.4 Replication cycle of coronaviruses

Members of the subfamily *Coronavirinae* undergo similar replication, transcription, and translation mechanisms except for the specific tissue tropism of an infected animal host. The replication cycle is as depicted in **Figure 1.8**, with the replication cycle being initiated by the attachment of the virus via the spike protein to a susceptible host cell. Tissue tropism is exclusively determined by the S protein-receptor interaction (Millet & Whittaker, 2015). The majority of CoVs utilize peptidases as their cellular receptors. The AlphaCoVs utilize aminopeptidase N (AMN) as their receptors, except HCoV-NL63 which utilizes angiotensin-

converting enzyme 2 (ACE2) (Jonsdottir & Dijkman, 2016). Upon viral attachment, acid dependent proteolytic cleavage facilitates virus entry into the host cell cytosol by separating the receptor-binding domain and the fusion domain of the spike protein, evoking a structural change that facilitates the fusion of the viral membrane and the host cell membrane (Belouzard et al., 2009; Fehr & Perlman, 2015; Millet & Whittaker, 2015).

Prior to RNA replication and transcription, replicase protein expression takes place. The expression of this protein is coded by the ORF rep1a and rep1b gene resulting in the expression of the polyprotein pp1a and pp1ab with the aid of the host cell's ribosomes (Fehr & Perlman, 2015; Ziebuhr, 2005). The polyproteins pp1a and pp1ab contain the nonstructural proteins (nsp 1-11 and 1-16 respectively) which are subsequently cleaved to individual nonstructural proteins. Cleavage is done by viral proteases such as Papain-like proteases (PLpro) encoded by nsp3, and serine-type proteases encoded by nsp5 (Graham et al., 2008). Many of the nsps assemble into replicase-transcriptase complex (RTC) to create an environment suitable for RNA synthesis and RNA replication and transcription of sub-genomic RNA (Wang et al., 2020). Viral RNA synthesis precedes translation and assembly of the RTC, resulting in the formation of antisense genomic RNA and subsequent genomic RNA progeny. The resulting sub-genomic RNA serves as a template (mRNA) for the expression of structural and accessory genes (Denison et al., 2011; Fehr & Perlman, 2015). The structure of subgenomic RNA is such that they exist as a 3' co-terminal full-length viral genome that results in a set of nested RNAs. Within the 5' untranslated region (UTR) exists seven stem-loop structures while the 3' UTR contains a bulged stem-loop, pseudoknot, and hypervariable region all of which overlap and thus cannot be formed simultaneously. The three structures are proposed to regulate alternate stages of RNA synthesis (Brian & Baric, 2005; Goebel et al., 2007; Manfredonia & Incarnato, 2021; Staple & Butcher, 2005). The leader sequence and transcription regulatory sequence (TRS) segments fuse during the process of subgenomic RNA synthesis and are believed to occur on the discontinuous extension negative strand. The current replication model proposes that the RdRp (RNA Dependent RNA Polymerase) passes at one of the TRS bodies (TRS-B) following the pause, RdRp either continues elongation to the next TRS or switches to amplifying the leader sequence at the 5' end of the genome-guided by the complementarity of the TRS-B to the leader TRS (TRS-L) (Denison et al., 2011; Sola et al., 2005). The strand switching ability of RdRp facilitates recombination in coronaviruses which may be homologous or non-homologous, and this forms the basis of viral evolution of coronaviruses (Fehr & Perlman, 2015; Wang et al., 2020).

Wang et al.,(2020) indicates that upon completion of replication and synthesis of subgenomic RNA, the structural proteins S, M and E proteins are translated. Thereafter, these proteins are

transported to the endoplasmic reticulum (ER) where they are then trafficked through the secretory pathway into the endoplasmic reticulum Golgi intermediate compartment (ERGIC). The viral genomes are encapsulated by the N protein, and bud into ERIC membranes containing the viral structural proteins resulting in the formation of mature virions. Despite not playing a crucial role during viral assembly, the S protein is transported to the ERGIC where it works together with the M protein which facilitates its incorporation into the virions. The resulting virions are released via exocytosis (Fehr & Perlman, 2015).

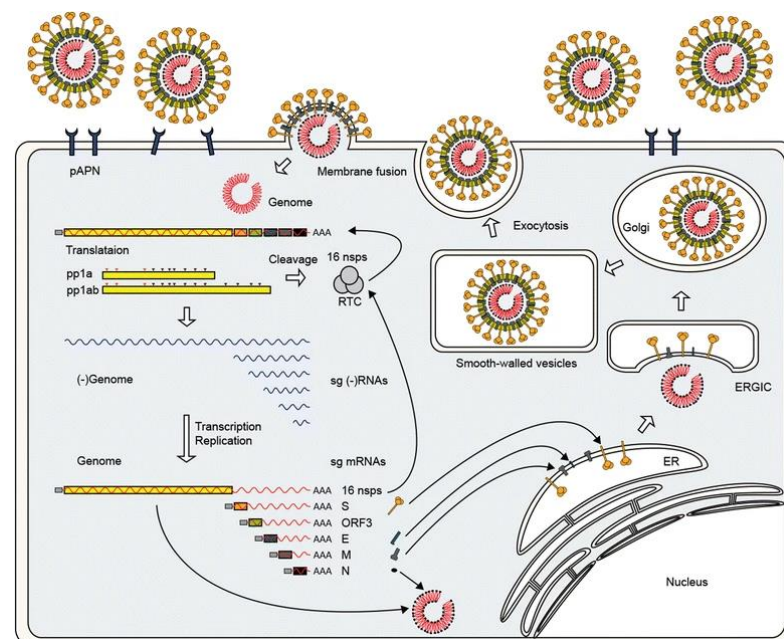


Figure 1.8: *Coronaviridae* replication cycle

Image by Changhee Lee. Adapted from "Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus" by Lee, C. (2015), Virol J, 12, 193. Licensed under CC BY 4.0.

Retrieved from <https://virology.biomedcentral.com/articles/10.1186/s12985-015-0421-2>

1.5.5 Pathogenesis of *Coronaviridae*

Genetic studies have classified AlphaCoV and BetaCoV to encompass coronaviruses that affect mammals including humans, while those of the DeltaCoV affect primarily birds (Marchenko et al., 2022; Q. Wang et al., 2022), with some spillover to mammals such as leopard cats, ferret-badger (Kong et al., 2022) and porcine (Li et al., 2014; Wang et al., 2014). Human coronaviruses (HCoVs) primarily infect the respiratory tract and gastrointestinal tract with most exhibited cases being common cold or severe cases such as bronchitis and/or pneumonia (Chen et al., 2020; Su et al., 2016). The first HCoV isolated in humans was the B814 in 1965 (Kaur et al., 2021), while other strains such as the HCoV-NL63 and HCoV-HKU1 were identified in 2004 (van der Hoek et al., 2004) and 2005 (Woo et al., 2005), respectively, though

they had been circulating within the human populations for a longer time. The latest coronaviruses to accidentally spill over from a zoonotic reservoir or intermediate hosts and then adapt to being maintained within human populations include SARS-CoV, SARS-CoV2 and MERS-CoV (Burki, 2020; Mulabbi et al., 2021).

Animal CoVs include those affecting swine such as the transmissible gastroenteritis virus (TGEV) (Valitutto et al., 2020; Yuan et al., 2021) which causes severe inflammation of the stomach and intestines; and porcine hemagglutinating encephalomyelitis virus (PHEV) (Mora-Díaz et al., 2019) that infects the nerve tissues. Bovine coronavirus (BCoV) primarily affects cattle but may be transmitted to other ruminants (Vlasova & Saif, 2021). The coronaviruses are of economic concern due to the losses incurred particularly in nations that primarily focus on animal production at a large scale. Other animal coronaviruses include feline enteric coronavirus (FCoV) detected in felines (cats and members of the cat family), murine hepatitis virus (MHV), canine coronaviruses, avian coronaviruses and dromedary camels' coronaviruses (Alluwaimi et al., 2020).

1.6 *Paramyxoviridae*

Paramyxoviridae is a family of enveloped RNA viruses of medical and veterinary importance as they cause respiratory, neurological, and systemic illnesses in humans and other animals (Outline, 2017). Paramyxoviruses that affect humans include measles virus, mumps, parainfluenza viruses and respiratory syncytial virus (RSV). The world health organization (<https://www.who.int/news-room/fact-sheets/detail/measles>) reported 120,000 measles related deaths in 2021, with the under 5 years age group being the predominantly affected. Paramyxoviruses also affect animals such as pigs and horses, infected with Nipah and Hendra respectively, with spillover to humans (Eaton et al., 2006). As characteristic of zoonotic outbreaks, health and economic constraints are trademark outcomes that follow. For example, the 1999 Nipah outbreak in Malaysia/Singapore caused substantial losses in the swine industry, with 40% mortality of 265 human cases reported (Chew et al., 2000; Chua, 2003).

1.6.1 Taxonomy

Paramyxoviridae is one of eleven families within the order *Mononegavirales* that is characterized to include viruses with a non-segmented single stranded negative-strand RNA genome. Within the family, there are four (4) subfamilies: *Avulavirinae*, *Metaparamyxovirinae*, *Rubulavirinae* and *Orthoparamyxovirinae*. The latter subfamily has eight (8) genera: the non-mammalian genera *Aquaparamyxovirus* and *Felavirus*, as well as the *Henipavirus*,

Jeilongvirus, *Morbillivirus*, *Narmovirus*, *Respirovirus* and *Salemvirus* genera (Kuhn et al., 2021; Rima et al., 2019). The classification of paramyxoviruses has proven challenging within the last decade notably with the *Jeilongvirus* (Rima et al., 2018), where initially, some of the jeilongviruses were reported to be morbillivirus related, but have since been re-classified as a separate genus. The taxonomy is summarized in **Figure 1.9** below.

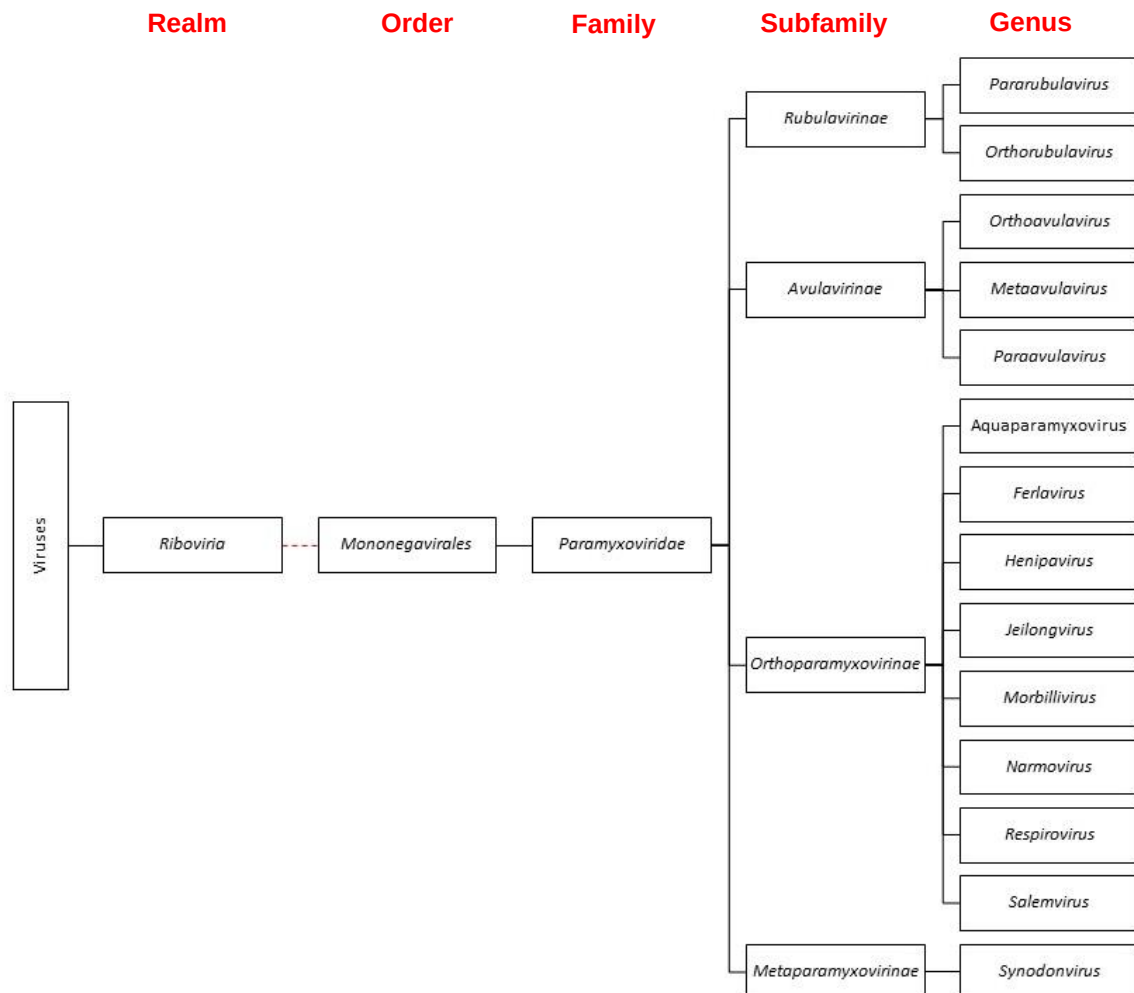


Figure 1.9: *Paramyxoviridae* taxonomy

1.6.2 Morphology and genomic structure

Paramyxoviruses (**PMVs**) are enveloped viruses that have two types of glycoproteins embedded on the surface; fusion protein and receptor binding proteins (RBPs). The latter glycoproteins vary according to the virus genus as either hemagglutinin (H), hemagglutinin neuraminidase (HN) or glycoprotein (G) (Rima et al., 2019). Their genome size ranges between ~15kb-20kb long and may be either pleomorphic or spherical depending on the virus type (Battisti et al., 2012). Genome organization includes structural protein-coding genes flanked by UTRs at the 5' and 3' ends of the ORFs. The genome encodes the nucleocapsid

(N) protein, matrix (M) protein, phosphoprotein (P), fusion (F) protein or receptor-binding proteins (RBPs), and the multifunctional large polymerase (L) protein (**Figure 1.10 a&b**). Short (10-13nt) conserved sequences known as the gene start and gene end signals are also found along the genome, and signals flank each protein-coding gene and are vital during transcription (Cox & Plemper, 2017).

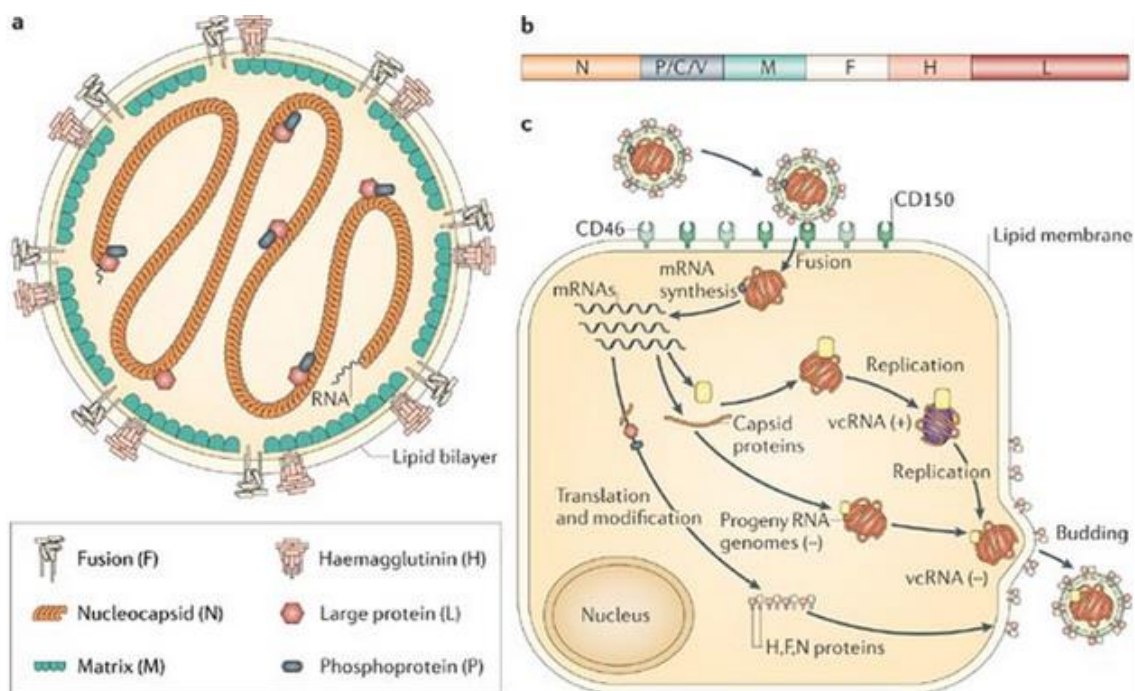


Figure 1.10: Structure, genome organization and life cycle of Measles virus

(a) Measles (Morbillivirus genus) virion structure showing key structural proteins: envelope (lipid layer), fusion protein (F), receptor binding protein (Haemagglutinin-H), matrix protein (M), nucleocapsid (N), phosphoprotein (P), and large protein (L). (b) Genome organization of the ~16kb viral genome. (c) Measles virus life cycle depicting stages of viral replication and infection. Used with permission of Springer Nature, from "Global Measles Elimination", William J. Moss et al., Nature Reviews Microbiology 4, 2006; permission conveyed through Copyright Clearance Center, Inc.

1.6.3 Structural Proteins and their function

The summary of the paramyxovirus structural proteins and their respective functions are described as reviewed by Battisti et al., 2012; Cox & Plemper, 2017; MacLachlan & Dubovi, 2017a; Morrison, 2003; Samal, 2008. These structural proteins vary in terms of size and play various roles in the life cycle of the virus and are summarized in **Table 1.3**.

Table 1.3: *Paramyxoviridae* virion protein structure and function

Protein	Size (kDa)	Structure	Function	Reference
Fusion (F)	~60	type 1 glycoproteins with amino-terminal signal sequence, hydrophobic transmembrane domain (TM) located near carboxyl terminus, and 25–30-amino-acid cytoplasmic domain (CT)	fusion of viral and host cell membranes	(MacLachlan & Dubovi, 2017b; Morrison, 2003)
Glycoprotein (G); Hemagglutinin (H); Hemagglutinin-neuraminidase (HN)	‡	transmembrane type II proteins comprised of N-terminal cytoplasmic tail followed by transmembrane domain, stalk, and globular head	G bind to protein receptors; HN bind to sialic acid receptors and subsequently cleave it, whereas H bind to sialic acid receptors without neuraminidase activity (sialic acid cleavage)	(Zaitsev et al., 2004)
Nucleocapsid protein (NP)	~50	Comprised of two globular domains, N-terminal domain and C-terminal domain which form positively charged core to encompass viral RNA	encapsulates viral genome	(Bloyet, 2021; Samal, 2008)
Matrix protein (M)	~40	built of one or two domains that have similar β -sandwich folds	viral assembly and budding process through interaction with the cytoplasmic tails of glycoproteins and NP	(Battisti et al., 2012)
Large protein (L)	~250	has conserved regions: RNA-dependent RNA polymerase (RdRp), poly-ribonucleotidyltransferase (PRNTase), connecting domain (CD), methyltransferase (MTase), and C-terminal domain (CTD)	viral enzymatic activities (transcription and replication) with assistance of the P protein	(Abdella et al., 2020)
Phosphoprotein (P)	-	domains: N-terminal, central oligomerization (OD), and C-terminal X domain (XD)	cofactor for L protein during synthesis of genomic and antigenomic RNA, and synthesis of individual mRNAs during transcription	(Abdella et al., 2020)

(‡) denotes that the specific protein size is unreported

1.6.4 Replication cycle of paramyxoviruses

Understanding of the replication and transcription in paramyxoviruses is based on studies on Sendai virus (SeV) and other non-segmented negative-strand RNA viruses such as a respiratory syncytial virus (RSV) and vesicular stomatitis virus (VS) from the *Rhabdoviridae* family (Abdella et al., 2020). The replication cycle is depicted in **Figure 1.10(c)** and is initiated by the attachment of the virus via the receptor binding proteins (RBPs). The type of viral RBP used in the attachment process depends on the virus subfamily and/or genus (Rima et al., 2019). In the case of the subfamily *Orthoparamyxovirinae* these may be either hemagglutinin (H) as seen in the *Morbillivirus*; glycoprotein (G) for *Henipavirus*, *Narmovirus*, *Salemvirus*, and *Jeilongvirus* (Aguilar et al., 2016; Dong et al., 2020); or hemagglutinin neuraminidase (HN) for

genera *Respirovirus*, *Aquaparamyxovirus*, and *Ferlavirus* (Lin & Richardson, 2016). The subfamilies *Avulavirinae*, *Metaparamyxovirinae* and *Rubulavirinae* also utilize the HN protein during attachment (Rima et al., 2019). Following attachment, virion entry into the host cell's cytoplasm is facilitated by the fusion (F) protein. The F protein facilitates the fusion of the virion envelope with the host cell's cell membrane. Upon successful fusion, the nucleocapsid is released into the cytoplasm (Bossart et al., 2013; Navaratnarajah et al., 2020).

Replication and transcription are facilitated by the viral polymerase complex (L and P protein) and its associated viral genome (Bloyet, 2021). Primary transcription (mRNA synthesis) takes place upon the release of the complex into the host cell cytoplasm (MacLachlan & Dubovi, 2017b). During transcription, the structure of the non-segmented -ssRNA is such that it has transcriptional elements that encode for 7-11 proteins, with each element encoding a single specific mRNA (Rima et al., 2019). The transcriptional elements vary according to the viral genus, and furthermore the abundance of mRNA generated decreases from the 3' to 5' (N protein encoding transcript abundant; L protein transcripts are least abundant) (Payne, 2017b). Transcription follows the start-stop process, where *gene start* directs the initiation of mRNA synthesis, whereas *gene stop* directs the termination of synthesis of an mRNA while also signaling the incorporation of the poly-A cap (Noton et al., 2019; Wignall-Fleming et al., 2019). The polymerase initially recognizes the leader (*le*) promoter on the 3' end of the genome and reads along until it encounters the *gene start* which signals the synthesis of a specific mRNA (Bloyet, 2021; Noton & Fearn, 2015). Conversion of mRNA to viral proteins also occurs within the host cell cytoplasm using the host cells ribosomes. The level of viral proteins synthesized correlating to the abundance of mRNA generated during transcription (Payne, 2017b).

Initiation of replication takes once cytoplasmic N proteins reach a certain threshold (MacLachlan & Dubovi, 2017b). The abundance of this particular protein causes the polymerase to switch from transcription to replication (Plumet et al., 2005; Takimoto & Portner, 2004). The polymerase recognizes the *le* promoter and synthesizes antigenomic RNA by ignoring the gene start-stops signals within the genome (Noton & Fearn, 2015). Antigenomic RNA serves as intermediate that will be used to synthesize more genomic RNA during replication, due to the presence of the trailer (*tr*) signal on the 3' end recognized by the polymerase. During RNA synthesis, encapsidation concurrently takes place and is also dependent on the cis-elements found within the genome (Noton & Fearn, 2015).

Viral assembly and release vary amongst the members of the *Paramyxoviridae* family. Viral assembly occurs as viral proteins and genomic RNA are transported to the plasma membrane

(Takimoto & Portner, 2004). Post translation modification of F and RBP proteins takes place and subsequently transported to the host cell membrane (Quarleri et al., 2022; Rima et al., 2019). Despite this, three structural proteins facilitate viral assembly and release. These are the surface RBPs, the ribonucleoprotein (RNP) and the matrix proteins (Harrison et al., 2010). El Najjar et al., (2014) and Battisti et al., (2012) reveal that the matrix proteins are synthesized in higher levels unlike the other structural proteins and interact with both the surface glycoproteins and RNPs during viral assembly, and additionally the host cell plasma membrane during release of mature viral progeny. In the case of HN using viruses such as parainfluenza and mumps virus, the neuraminidase activity ensures there is no aggregation of newly formed viral particles (Forgione et al., 2020). Budding and release of virions occur at defined sites, and these sites are dictated by viral glycoproteins' interactions with the host cell membrane lipids. The newly formed viral particles are released from the host cell via budding, where they can infect new host cells thus continuing the replication cycle (Ke et al., 2018).

1.6.5 Pathogenesis of *Paramyxoviridae*

PMVs affect both animal and human hosts and are usually localized within the respiratory tract specifically the epithelial cells but may also affect the central nervous system (CNS) (Navaratnarajah et al., 2020). The diseases caused by viral pathogens of the *Paramyxoviridae* family have heavy economic implications, due to high morbidity and mortality rates, on a local or national scale (Thibault et al., 2017; Vigant & Lee, 2011). Wang et al. (2021) reported by 2020, global human parainfluenza infections to be ~18 million, with ~35 000 deaths associated with the infections. **PMVs** of public and animal health concern include Newcastle disease virus (NDV) (which affects poultry and other avian species), bovine respiratory syncytial virus (cattle), and bovine parainfluenza virus 3 (affects cattle, and sheep) (reviewed by Samal, 2008). **PMVs** may be zoonotic in nature, and spillover into the human population where they are highly pathogenic. These include the henipaviruses, Nipah virus and Hendra virus which affect pigs and horses, respectively (Eaton et al., 2006; Thibault et al., 2017). Nipah virus has a 40-75 mortality rate since its first report in 1999 (Singh et al., 2019; Thakur et al., 2022). In terms of the economic implications associated with the outbreak, losses of up to US \$582 million as a result of pig culling (~1.1 million animals) for disease management (Smith et al., 2019). Though Hendra virus infections are sporadic, mortality rates upon infection are 80 and 60% in horses and humans, respectively (Yuen et al., 2021).

1.7 Studies from Sub-Saharan Africa

Bat-borne viruses including coronaviruses and paramyxoviruses have been identified globally in regions where bats are found. A study by Brierley et al. (2016), investigated the factors and

patterns of **viruses shared between bats and humans** on a global scale. **Figure 1.11** shows the extent of viral shedding, showing a significant proportion of bat-human shared viruses being found in Sub-Saharan Africa. This region has thus become a possible hotspot for the next outbreak of zoonotic disease of bats origin. In addition, the study also used modelling to predict how drivers of viral shedding such as human behaviour and demography facilitate viral richness and an opportunity for cross-species transmission. Notably, several bat species have been associated with various viruses globally, including in Sub-Saharan Africa as discussed in subsequent paragraphs.

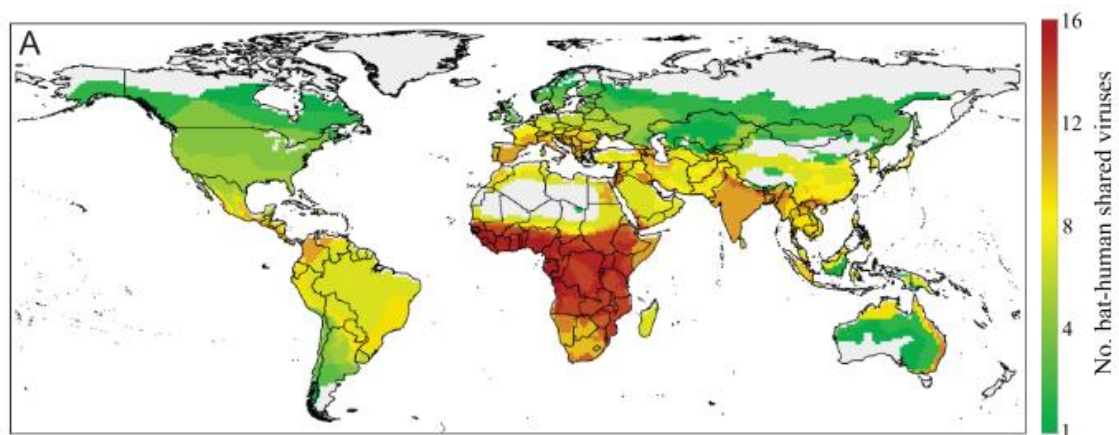


Figure 1.11: Patterns of bat-human virus sharing based on reported studies.

Used with permission of University of Chicago Press – Journals, from Quantifying Global Drivers of Zoonotic Bat Viruses: A Process-Based Perspective, Liam Brierley, Maarten J. Vonhof, Kevin J. Olival, Peter Daszak, and Kate E. Jones, *The American Naturalist* 2016 187:2, E53-E64; permission conveyed through Copyright Clearance Center, Inc.

Geldenhuys et al., (2021) gives an overview of biosurveillance of coronaviruses in Africa, indicating Central and Eastern Africa being regions where most studies were done. **The studies used wide range of biological material from bats found within various roost sites such as trees, human dwellings, and primarily caves.** Additionally, in some studies, sampling was not limited to bats as non-human primates and rodents were also included in sample collection. Identified coronaviruses include two subgenera of the *Alphacoronavirus*, *Setracovirus* (HCoVNL63-related viruses) and *Duvinavirus* (HCoV229E-related viruses). Other reported subgenera are classified under the *Betacoronavirus* include *Hibecovirus*, *Nobecovirus*, *Merbecovirus* (MERS-related viruses); and *Sarbecovirus* (SARS-related viruses). The majority of **viruses reported in these studies** have been **isolated** from Kenya. The method of detection in most studies was conventional PCR, with the starting biological material being samples from various anatomical sites in bats, such as rectal swabs, oral swabs, urine, blood, and gastrointestinal tissues. **Fecal** matter represents a substantial proportion of samples (70%) used in these studies. Geldenhuys et al., (2021) **further delves into the bat-host specificity of these coronaviruses, highlighting findings such as the presence of**

Sarbecoviruses in horseshoe bats; *Merbecovirus* in bats of the Vespertilionidae family, while Nobecoviruses are found within fruit bats.

The first report of paramyxoviruses in Africa was reported in Ghana when *Henipavirus*-related antibodies were detected in the sera obtained from African straw coloured fruit bat (Hayman et al., 2008). Molecular studies were further done by Drexler et al., (2009) for the detection of viral RNA in fecal samples (Drexler et al., 2009). Further surveillance of bat-borne paramyxoviruses showed their presence in fruit bats and insectivorous bats, including Egyptian rousette bat, Little collared fruit bat, Gambian epauletted fruit bat and hammer-headed bat (Drexler et al., 2009; Maganga et al., 2014; Mortlock et al., 2015; Suu-Ire et al., 2022). **Urine has predominantly been used and it is the most suitable sample for PMV surveillance in bats.** Despite the detection of paramyxoviruses in several bat species in Africa, some of which may roost in close proximity to humans, only one case of human infection has been reported in Uganda associated with Sosuga virus associated with Egyptian rousette bats (Albariño et al., 2014). Notably, paramyxoviruses have been detected in intermediate hosts such as pigs (Hayman et al., 2011). Serological studies have shown the presence of antibodies against paramyxoviruses in animals and humans, in samples collected from Ghana and Cameroon (Pernet et al., 2014). These findings indicate potential of spillover into animal and human populations, with risk factors such as the slaughter and consumption of bat meat (Kasso & Balakrishnan, 2013; Pernet et al., 2014) likely to increase the incidence rates. Despite the studies highlighted in Sub-Saharan Africa, information gaps are present particularly for Botswana as such surveillance studies have not been carried out.

In southern Africa, surveillance of coronaviruses and paramyxoviruses has been carried out in countries such as Zambia (Muleya et al., 2014), Mozambique (Hoarau et al., 2021; Léa Joffrin et al., 2020), Zimbabwe (Bourgarel et al., 2018; Chidoti et al., 2022), Eswatini (Shapiro et al., 2021) and extensively in South Africa (Geldenhuys et al., 2013, 2018; Ithete et al., 2013; Mortlock et al., 2015). The reported bat species that harbor these viruses within the region include **Angolan free-tailed bat, Straw-colored fruit bat, Sundevall's roundleaf bat, Egyptian fruit bat, Horseshoe bats, long-tailed serotine bat, African trident bat, Egyptian slit-faced bat** (Geldenhuys et al., 2013; Hoarau et al., 2021; Léa Joffrin et al., 2020; Mortlock et al., 2015; Muleya et al., 2014). Few of the aforementioned bat species have been reported to inhabit various regions of Botswana. Monadjem et al., (2021) has described some bat species that have been identified in Botswana, most of which have been identified within the northern region, some appearing sporadically in the southern region. The species identified includes four families from the Pteropodiformes suborder (Rhinolophidae, Pteropodidae, Hipposideridae, and Rhinonycteridae), and five (5) families from the suborder

Vespertilioniformes including Molossidae, Emballonuridae, Miniopteridae, the widely distributed Nycteridae, and Vespertilionidae which has the most identified species (Ferguson et al., 2010). The roosting behavior of the bats identified varies according to species, but they have been known to roost in caverns, crevices of rocky outcrops, dense foliage, burrows, building roofs and caves.

Bats are known to inhabit the Gcwihaba cave system, an ecotourism attraction found in the north-western part of Botswana. Additionally, the northern region of Botswana, comprises of other tourist attractions including the Okavango delta, Chobe River, and the Aha hills which are ≈50 km from the Gcwihaba caves. As bats can migrate over long distances and interact with other bats elsewhere, and other wildlife directly or indirectly, transmission of viruses between bats in different geographical regions is highly likely. Therefore, bats in Botswana may harbor viruses that are endemic in bats studied in South Africa, Zambia and Zimbabwe (Geldenhuys et al., 2013, 2021; Muleya et al., 2014). Despite Botswana lagging behind with research on zoonotic viruses, a framework of the necessary infrastructure has been proposed as per the Botswana National Research Plan prepared in 2005 ([Microsoft Word - BNRST Final Report December 2005.doc \(researchictafrica.net\)](#)).

Young and Olival (2016) compiled a report specifically summarizing the patterns of infection of viral families within different bat families, and the factors that increase the likelihood of viral discovery including specimen type and detection method. The report primarily used data from 93 studies published between 2007-2013, with the findings for prevalence for the virus and bat host family discussed extensively. Additionally, findings based on the specimen type used in the studies indicate viral discovery with the use of non-invasive methods such as fecal collection (used in this thesis) showed the same degree of positivity as invasive sample collection (such as blood or tissue). Based on the report by Young and Olival, and the preliminary nature of the current investigation, fecal samples were collected and used for this thesis.

1.8 Study rationale

The global health security (GHS) index has ranked Botswana 108 of 195 countries based on its capability and preparedness to manage emerging infectious diseases (EID) (Index, 2021). Biosurveillance of EID has been more streamlined to (i) diseases within the agricultural sector such as FMD (Batlang et al., 2014), due to the exportation of beef to the European Union; (ii)

and chronic diseases such as TB and HIV (Baik et al., 2020) which are infectious diseases of public health concern. Surveillance of viral zoonotic diseases is non-existent in Botswana with few studies done on bacterial zoonoses, including *Leptospira interrogans* and *Brucella* spp. in wildlife (Alexander et al., 2012; Jobbins et al., 2014). Paramyxoviruses and coronaviruses have been identified in bat species within the SADC region (as shown in section 1.7), with selected bat species being associated with these viruses, found to inhabit various habitats in Botswana.

In the last two decades, the world has witnessed the emergence and re-emergence of viral zoonoses that were either confirmed or suspected to be harbored by bats. Bats are considered natural hosts for the genetic diversities of various viral groups that include emerging viruses such as coronaviruses and paramyxoviruses. These virus families have been identified using molecular-based tools and documented in Botswana's neighboring countries: South Africa, Zambia, and Zimbabwe. The risk of virus infection of animal origin exists in Botswana and in the SADC region, given human encroachment into wildlife habitat, as well as using caves for ecotourism. As this is the first study to investigate bat viruses in Botswana, the data that would be generated in this pilot study would be useful for future biosurveillance studies, as well as facilitate prevention and control initiatives including framework for the construction and implementation of surveillance of viruses within bats, other wildlife, and human populations. Furthermore, the data obtained will provide the basis for population-wide immunological studies to evaluate who has been exposed through direct or indirect contact with bat populations.

1.9 Aim and Objectives

The aim of this research was to determine the presence or absence of viral RNA from coronaviruses and paramyxoviruses in fecal samples of bats inhabiting Gcwihaba caves.

Specific Objectives

- To extract viral genetic material and amplify conserved target sequences of the *Coronaviridae* and *Paramyxoviridae* families from environmentally collected bat fecal samples.
- To sequence the amplified partial gene sequences using Sanger sequencing
- To determine the evolutionary relationship through phylogenetic analysis to those that have been previously detected within the southern Africa region.

Chapter 2: Methodology

2.1 Sample collection

Fecal samples (n=173) were collected in June (dry) 2020 and March (wet) 2021 (to allow for a more comprehensive range of bat species and viral activity) within the Gcwihaba caves. **The sampling was performed once in both sampling months.** The samples were collected by laying out 2 m by 2 m plastic sheets under randomly selected mini colonies (**Figure 2.1**). **Full diversity of bats inhabiting the cave is unknown, but based on previous report by Monadjem et al., (2021) on bat distributions in the northern region of Botswana, the following families were expected: Rhinolophidae, Nycteridae, Hipposideridae, Miniopteridae and Rhinonycteridae.** By visual inspection of characteristic morphological features, the dominant bats **were** noted for each mini-colony from which samples were collected. The sheets were left for at most 12 hours to allow **fecal** droppings to accumulate. **Traces of urine were noted on the sheets, thereby possibly contaminating the fecal matter.** Fecal droppings were collected with 70 ethanol-sterilized tweezers and transferred into 1.5 ml cryovials containing either 1 ml of **1X** DNA/RNA Shield (Zymo Research, USA) or RNA/*later*[™] stabilization solution (Invitrogen[™], Canada). **For each sample collected, the tweezers were sterilized. To avoid cross-contamination, fecal samples were carefully collected from areas distributed across the sampling sheet to create distinct pools, reducing the likelihood of obtaining multiple positive pools from a single bat's droppings.** The vials were filled in an approximated 1:1 ratio (1000ml: ~1000mg), resulting in a homogenized sample. The samples were then stored at room temperature for transportation, till they were stored at –20 °C. **Table 2.1** shows the details **of** the sample collection.

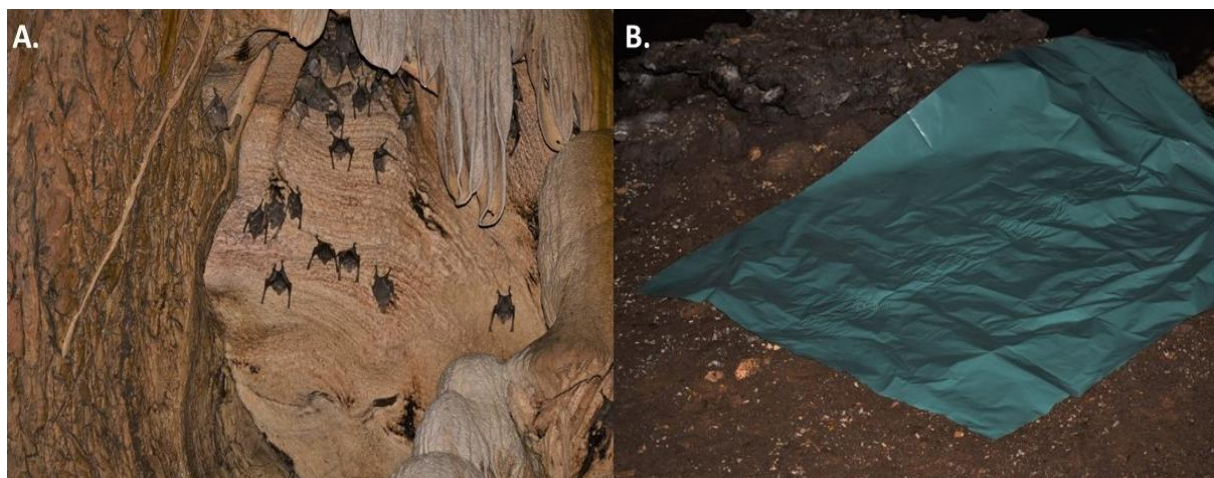


Figure 2.1: Sample collection with Gcwihaba cave.

A) Bats roosting on carved ceiling. **B)** Plastic sheets placed under selected roosting bats

Table 2.1: Samples collected and the respective bat species

Month of collection (season)	Bat species *	Number of fecal samples collected †
June 2020 (dry)	<i>Rhinolophus</i> spp.	14
	<i>Rhinolophus</i> spp.	34
	<i>Nycteris</i> spp.	51
March 2021 (wet)	<i>Rhinolophus</i> spp.	6
	<i>Hipposideros</i> spp.	27
	<i>Rhinolophus</i> spp.	41
Total		173

† *Rhinolophus* spp. appears twice with different sample numbers as samples were collected from separate sheets. The fecal samples gathered were a representation of the fecal material found on each respective sheet.

* bat species were identified virtually based on morphological characteristics

2.2 RNA/DNA extraction

Nucleic acid extraction was carried out in the BSL3 at the Centre for Viral Zoonoses at the University of Pretoria using Quick RNA/DNA Miniprep kit (Zymo Research, USA) as per manufacturer's instructions. The samples were thawed to room temperature and ≈ 100 mg of sample was measured out into a clean 1.5 ml microcentrifuge tube. RNA Lysis buffer (supplied with kit) was added to the tube at 600 μ l and mixed by vortexing. The tube's contents were spun down in a centrifuge at 16,000 x g for 30 seconds. The resulting supernatant was transferred into a clean 1.5 ml microcentrifuge tube. The supernatant was then transferred onto a Spin-Away™ filter (supplied with kit) placed in a collection tube and spun down at 16,000 x g. Ethanol (100 v/v) was added at 600 μ l to the resulting flow through containing RNA and mixed by pipetting up and down. The mixture was then transferred onto Zymo-Spin™ IICR Column (supplied with kit) placed in a clean collection tube and spun down at 16,000 x g for 30 seconds. The flow-through in the collection tube was discarded, and 400 μ l of DNA/RNA Prep buffer (supplied with kit) was added to the column and spun down at for 30 seconds at 16,000 x g. The flow-through was discarded, 700 μ l DNA/RNA Wash Buffer (supplied with kit) was added to the column and the centrifugation step was carried out at the same settings. The flow-through was discarded and the wash step was repeated with 400 μ l of DNA/RNA Wash Buffer with an extended centrifugation time of 2 minutes. The column was then transferred to a clean nuclease-free 1.5 ml microcentrifuge tube and RNA was eluted using 50 μ l DNase/RNase-Free Water added directly to the column and centrifuging for 30 seconds. The eluted RNA was stored at -80 °C till required for cDNA synthesis.

2.3 Random cDNA synthesis

All reagents used for the synthesis were obtained from Thermo Fisher Scientific, USA, unless otherwise stated. Synthesis of cDNA was carried out by preparing two reaction mixtures (A and B) that were later combined. Mixture A contained 1 µl of random hexamer primers (Integrated DNA technologies, USA) at a working concentration of 100 ng/µl, 1 µl of 10 mM dNTPs, 6 µl nuclease free water and 5 µl of total extracted RNA. The mixture was then incubated at 65°C for 5 minutes in a SimpliAmp Thermocycler (Applied Biosystems, Thermo Fisher Scientific, USA). The second mixture, B, was prepared using SuperScript™ IV reverse transcriptase (Invitrogen™, Thermo Fisher Scientific, Canada). The mixture contained 1 µl of 0.1 M dithiothreitol, 4 µl of 5X SuperScript IV First Strand Buffer (both supplied with the reverse transcriptase kit), 1 µl of 200U SuperScript™ IV Reverse transcriptase, and 1 µl of 40 U/µl Ribolock RNase inhibitor. The resulting 7-µl mixture was added to the pre-incubated mixture A. To synthesize cDNA, the cocktail (mixtures A and B) was then incubated at the following thermocycler settings: 23°C for 10 minutes, 50°C for 30 minutes, and 80°C for 10 minutes. The synthesized cDNA was then stored at -80°C till used for gene specific amplifications.

2.4 Polymerase chain reaction (PCR) assays

The molecular detection of the subfamily *Orthocoronavirinae* (specifically the AlphaCoV and BetaCoV) was done based on the conserved sequences of the RdRp gene. For the detection of paramyxoviruses, two assays (RMH and SCR heminested RT-PCR) were employed for the detection of viruses in the genera belonging to the *Orthoparamyxovirinae* subfamily with samples tested with at least one of the assays. The assays targeted the polymerase gene (L gene). The summary of the primers used for the detection of each subfamily are indicated in Table 2.2 below.

Table 2.2: Primer sequences and their respective assays

Subfamily	Primer name	PCR Round used	Sequence 5'-3'	Final amplicon size (bp)
<i>Orthocoronavirinae</i>	αβ_For	P1	ATGGGHTGGGAYTAYCCHAARTGYGA	265
	Sarbeco_ForP1	P1	GGTTGGGAYTAYCCHAARTGYGA	
	Sarbeco_ForP2	P2	GAYTAYCCHAARTGTGAYAGA	
	β-For	P2	TAYGTIAARCCWGGHGGIAC	
	α_For	P2	TYTAYITIAARCCWGGTGG	
	α_Rev	Both	CCRTCATCWGAIRDATCATCAT	
	β_Rev	Both	CATCRTCASDIARDATCATCAT	
	Sarbeco_Rev	Both	CCRTCATCACTHARWATCAT	
<i>Orthoparamyxovirinae</i>	RMH-F1	P1	TCITTCCTTTAGAACI TTYGGNCAYCC	494
	RMH-F2	P2	GCCATATTTTGTGGAATAATHATHAAYGG	

	RMH-R	Both	CTCATTTTGTAGTC ATYTTNGCRAA	
	SCR-14F	P1	ATGATGAARGGNCATGC	~200
	SCR-16F	P1	AARGGNCATGCHHTNTTCTG	
	SCR-26R	P1	GCYTTTRTCYTTTCATRTACAT	
	SCR-25R	P2	TTCATRTACATDGTNAGRTC	

*Degenerate codes used: I- Inosine, D- A/G/T, H- A/C/T, R- A/G, S- G/C, W- A/T, Y- C/T.; P1=Round 1 and P2=Round 2

2.4.1 Paramyxovirus heminested PCR

2.4.1.1 RMH heminested PCR

A standard reaction mixture for the first round PCR reaction for the *Respiro-Morbilli-Henipavirus* (RMH) assay was prepared using 31.05 µl nuclease free water, 4 µl of 25 mM Magnesium chloride (Thermo Fisher Scientific, USA), 5 µl of 10X Dream Taq buffer, 2.2 µl of 10mM dNTP mix, 1.5 µl of 10 pmol RMH-Reverse primer, 1 µl of 10 pmol RMH-Forward primer, and 0.25 µl Dream Taq Polymerase. A 5-µl sample cDNA was added to the resulting 45-µl reaction mixture. Nuclease-free water was added as the negative control and canine distemper virus RNA (CDV), a *Morbillivirus*, was used as a positive control in separate reactions with a similar reaction mix. For the first round PCR, the reactions were transferred into the SimpliAmp thermocycler for amplification with the following settings: 94°C for 2 minutes, 40 cycles of 94°C for 15s, 48°C for 30s, and 72°C for 30s. Final extension was carried out at 72°C for 7 minutes and the reaction held at 4°C. The second round (nested round) reaction mix was prepared by adding 36.55 µl nuclease-free water, 5 µl of 10X Dream Taq buffer, 2.2 µl of 10mM dNTP mix, 1.5 µl each of 10 pmol forward and reverse primers (see **Table 2.2**), and 0.25 µl DreamTaq polymerase resulting in a final mixture volume of 47 µl. 3 µl of first-round product was added to the 47-µl reaction mixture. Amplification settings used were identical to those of round one PCR.

2.4.1.2 SCR nested RT-PCR

Inconclusive RMH tested samples (n=22) were re-tested using the SCR assay for the detection of paramyxoviruses of the Orthoparamyxovirinae subfamily. Both assays target the same gene region and share similar sensitivities. As such, viral sequences obtained could thus still be analyzed collectively. For the SCR first-round reaction, a standard 50 µl reaction comprised of 14 µl nuclease free water, 25 µl 2X DreamTaq Green master mix (Thermo Fisher Scientific, USA), 3 µl of 10pmol SCR14F and SCR26R primers (as indicated in **Table 2.2**) each, and 5 µl randomly primed cDNA initially prepared for RMH assay use. The cyclic conditions for the first-round reaction were: 2 minutes at 95°C for initial denaturation, followed by 40 cycles of 30s at 95°C, 30s at 47°C, 1 minute at 72°C, and a final elongation at 72°C for 10 minutes. For the second (nested) round, the cyclic conditions and reaction volume

remained unchanged. However slight changes made for the standard 50 µl prepared, including the primers used (SCR16F and SCR25R), 16 µl nuclease free water, and 3 µl first round PCR product.

2.4.2 Pancoronavirus heminested PCR

A standard reaction mixture for the first round of the assay was prepared by mixing 32.75 µl nuclease-free water, 10 pmol at 1 µl αβ_For forward primer, 10 pmol at 1 µl Sarbeco_For forward primer, 10 pmol at 2 µl α_Rev reverse primer, 10 pmol at 1 µl β_Rev reverse primer, 10 pmol at 1 µl Sarbeco_Rev reverse primer, 2 µl of 10 mM dNTPs, 5 µl of 10X Dream Taq buffer (Thermo Fisher Scientific, USA), and 0.25 µl of 5 U/µl Dream Taq Polymerase giving a final volume of 46 µl. Then 4 µl of cDNA was added to the reaction mixture. For the negative and positive controls, Nuclease-free water and NL63 cDNA were added, respectively, instead of the sample cDNA. Reaction mixtures were amplified under the following conditions: 94°C for 1 minute, 35 cycles of 94°C for 30s, 48°C for 30s, and 72°C for 45s. Final extension was carried out at 72°C for 7 minutes and the reaction held at 4°C. The nested round of the assay was carried out using the round 2 (P2) forward and reverse primers (indicated in **Table 2.2**). The forward primers were added at a volume of 2 µl α_For, and 1 µl each for the β_For and Sarbeco_ForP2 primers. The reverse primers were added at 1 µl α_Rev primer and 0.5 µl each of the β_Rev and Sarbeco_Rev primers. Nuclease water volume was adjusted to 33.75 µl giving a reaction mixture of 47 µl. The first-round product was added to the second-round standard reaction at 3 µl and mixed before carrying out the nested PCR round. Amplification settings used were identical to those of the first PCR round.

2.5 Gel electrophoresis and DNA gel purification

Analyses of heminested PCR products was carried out by horizontal agarose gel electrophoresis. A 1.5 agarose gel was prepared by dissolving the required amount of CLS-AG 500 LE Agarose Multi-Purpose Agarose (Cleaver Scientific, UK) in 1X TAE buffer (Thermo Fisher Scientific, USA). The agarose was supplemented with 2.5 µL SmartGlow™ Safe Green Pre-Stain to 50 ml agarose to visualise nucleic acids. DNA Loading dye (6X) was added at 5 µl for the full 50 µl nested reaction and transferred to the gel wells for electrophoresis. To facilitate the approximation of the amplicon size, Generuler 100bp DNA ladder (Thermo Fisher Scientific, USA) was added in the first well. The samples were run on gel in 1X TAE buffer, at 120 V for 40 minutes. The gel was visualized using the Gel Doc™ XR+ Imaging system (Bio-Rad, USA). The expected amplicon sizes for positive samples were 494 bp and 265 bp for the RMH and coronaviruses assays, respectively.

For positive samples identified with the Gel Doc system, the bands were excised from the gel and transferred into a pre-labelled 1.5-ml microcentrifuge tube with the aid of a Slimline™ UV transilluminator (Spectroline, USA). The weight of the tube with the excised band was noted. Gel purification was carried out using the Zymoclean™ Gel DNA recovery kit (Zymo Research), as per manufacturer's instruction. The excised band was dissolved 3 times the weight of the excised band at 55°C using a heating block. Thereafter, the dissolved contents were transferred onto a spin column, spun down at 12,000 x g, and the flow through was discarded. DNA wash buffer (200 µL) was then added to the column, and spun down again at 12,000 x g. The flow through was discarded, and the wash step was repeated. The column was then transferred to a new pre-labelled 1.5-ml microcentrifuge tube. Finally, 10 µl DNA elution buffer (supplied with the gel recovery kit) was added directly to the column, incubated for 1 minute, then spun down at 12,000 x g. For the determination of successful gel purification process, 1 µl of the purified product was ran alongside molecular ladder with loading dye added at a 1:1 ratio, with the electrophoresis gel visualized in the Gel Doc XR+ imaging system. An estimate of the concentration of the purified DNA was determined for use in subsequent sequencing reactions.

2.6 Sequencing and clean-up

Sequencing of the purified product was carried out using BigDye™ Terminator v3.1 sequencing kit (Applied Biosystems, USA). For each reaction, 5.4 µl nuclease-free water, 2.1 µl of 5X Sequencing buffer, 1 µl of 3.2 pmol specific primer, and 0.5 µl Big Dye Terminator v3.1 were added to give a 9 µl reaction mixture. Finally, 1 µl of DNA (~3-10ng for CoV and RMH assays, 1-3 ng for SCR assay), was added to give a sequencing reaction mixture of 10 µl. The mixtures were incubated in the SimpliAmp thermo cycler with the following cycling conditions: 96°C for 1 minute, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The reaction was held at 4°C.

Cleaning up of the BigDye sequencing PCR products was done to remove unincorporated labelled dNTPs and excess reagents. This was done using the ZR DNA Sequencing Clean-up kit™ (Zymo Research, USA). Sequencing buffer was added at volume of 240 µl per 10 µl sequencing reaction. The mixture was mixed via pipetting, transferred to Zymo-Spin™ IB Column placed in a collection tube and spun down in a centrifuge at 16,000 x g for 30 seconds. Sequencing Wash Buffer (300 µl) was then added to the column and spun down at the same speed. The columns were transferred to clean 1.5 ml microcentrifuge tubes, and 10 µl of nuclease free water was added directly to the column and spun down at 16,000 x g for 15 seconds to elute the DNA. The eluted DNA was then sent for Sanger sequencing. Sanger

sequencing was performed at the sequencing facility at the Faculty of Natural and Agricultural Sciences, University of Pretoria using the ABI 3500xl sequencer (Thermo Fisher Scientific, Carlsbad, USA).

2.7 Sequence editing and review

Following Sanger sequencing, the sequence chromatogram for the reverse and forward sequence files for each positive sample was examined to determine the quality of the raw sequence files. BioEdit (Hall, 1999) was used to visualize the chromatogram to determine the degree of background noise, correct base calling and whether the sequence length is as expected. Where overlapping peaks were observed upon visualization of the sequence chromatogram, co-infection was hypothesized, and cloning was carried out (Cloning and colony PCR 2.8). For sequences where good resolution of peaks with low background noise, the raw sequences were submitted to NCBI nucleotide blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for confirmation of sequence identity.

The forward and reverse sequence files of which the sequence identity was confirmed were then manually edited using BioEdit. Manual editing included trimming the first 10-20 bases at the end and beginning of the sequence and remove ambiguous/degenerate bases for the forward and reverse sequence files according to the IUPAC nucleotide ambiguity code (<https://www.dnabaser.com/articles/IUPAC20ambiguity20codes.html>). Following manual editing, a consensus sequence was generated firstly by running an alignment of the forward and reverse-complemented reverse sequence using “ClustalW” function on BioEdit. The “Create consensus sequence” function of BioEdit was used on the alignment output resulting in a consensus sequence. Successful generation of a consensus sequence was indicated by the absence of mismatches upon assembly of the forward and reverse-complemented reverse sequence files following alignment. Finally, confirmation of the consensus sequence was done through submission on NCBI nucleotide blast using the “Highly similar sequences” option selected. The consensus sequences were used in the analysis as indicated in (section 2.9)

2.8 Cloning and colony PCR

For sequences that required cloning, the pGEM®-T Easy vector (Promega, USA) was used. The pGEM-T Vector, with a size of 3015 bp, was supplied at a concentration of 50 ng/μl. To determine the amount of DNA for the ligation reaction, the concentration of the PCR product was initially measured using Qubit (Invitrogen™) in triplicates, and the average was used with Equation 1 to get the amount to add. This was crucial for establishing the insert : vector ratio.

The initial reaction was performed at an insert : vector ratio of 3:1, given the characteristics of the pGEM-T Vector. Ligation reactions were prepared in 0.6-ml microcentrifuge tubes at 5 µl of 2X Rapid Ligation buffer for the standard reaction, positive control, and background control in three separate tubes. 1 µl of pGEM®-T Easy vector (supplied with the pGEM®-T Easy system), was added to each of these reactions. The PCR product volume was determined by **Equation 1** and added only to the standard reaction, while for the positive control, 2 µl of control insert DNA was added. 1 µl of DNA ligase at 3 Weiss units/µl was added into all three reactions (standard reaction, background, and positive control). Nuclease-free water was added to achieve a total volume of 10 µl in each reaction tube. The ligation reactions were left to incubate for 12 hours at 4°C.

$$\text{ng of insert to add} = \left[\frac{\text{ng of vector} \times \text{kb}}{\text{size of insert kb} \times \text{size of vector}} \right] \times \frac{\text{insert molar ratio}}{\text{vector molar ratio}}$$

Equation 1 Determination of insert DNA

Transformation was performed on duplicate LB (Luria Bertani) plates with Ampicillin (100 µl/ml) using JM109 High Efficiency competent cells (Promega, USA). To each of the pre-labelled plates, 100 µl of 0.1 M IPTG (Promega, USA) and 20 µl of 20 mg/ml X-gal (Promega, USA) were added and spread on each plate. The plates were then incubated at 37°C for 30 minutes to allow for absorption of the solutions into the media. The plates were equilibrated to room temperature. Aliquots of 50 µl JM109 competent cells were prepared in 1.5 ml microcentrifuge tubes. Ligation products were added to the competent cells at 2 µl per tube, flicked to mix the transformation culture, and incubated on ice for 20 minutes. Thereafter, heat-shock of the cells was performed at 42°C for 45 seconds, followed by incubation on ice for 2 minutes. SOC medium (Promega, USA) was added to the transformation culture at 950 µl. The tubes were then incubated for 90 minutes at 37°C with shaking at 150 rpm. For each sample, 100 µl of the transformation culture was plated on the LB/ampicillin/IPTG/X-Gal plates and incubated at 37°C for 24 hours.

Colonies containing the fragment of interest (white colonies) were selected for characterization with colony PCR using M13 primers by mixing with 50 µl nuclease-free water and centrifuging at 16,000 x g. The resulting supernatant containing the plasmid was used in colony PCR. A 45 µl standard reaction was prepared using 34.75 µl nuclease free water, 5 µl 10X Dream Taq buffer, 2 µl 10mM dNTPs, 1.5 µl of 10pmol M13 F (5'- GTAAAACGACGGCCAG – 3') primer, 1.5 µl of 10pmol M13 R (5'-CAGGAAACAGCTATGAC – 3') primer, and 0.25 µl 5U/µl Dream Taq polymerase. The plasmid supernatant was added to the reaction mix at 5 µl and mixed by pipetting. In the case of a negative control, 5 µl nuclease free was added to the 45 µl reaction mix. The tubes were then placed in the SimpliAmp thermocycler with the following

sequencing cycling conditions: 94°C for 5 minutes, 40 cycles: 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 90 seconds; final extension at 72°C and held at 4°C. The PCR product was analysed by agarose gel electrophoresis, where successful insertion of the insert into the pGEM®-T Easy vector was observed by a ≈700bp band, which was then excised for DNA purification and sequencing. Sequence editing and review was done as described in **Methodology** section 2.7 with additional removal of bases (recognized by the M13 primers) flanking the sequence of interest.

2.9 Phylogenetic analysis and sequence identity determination

Bayesian phylogenetic analysis was performed following sequence selection of representative coronavirus and paramyxovirus sequences from various host species including the bat genera identified in the study (*Rhinolophus*, *Nycteris* and *Hipposideros*) from NCBI GenBank (Available from: <https://www.ncbi.nlm.nih.gov/nucleotide/>). Information from the International Committee on Taxonomy of Viruses (ICTV) virus metadata resource containing up-to-date classified viruses (Walker et al., 2022) was also used to facilitate sequence retrieval. The full list of sequences included for analysis is shown in **Table 7.2** and **Table 7.4**. Alignment of sequences was performed using Clustal Omega (Sievers et al., 2011), and edited using BioEdit (v7.2.5) (Hall, 1999). **The alignments were trimmed to the same length, CoV MSA trimmed length 207 nucleotides, RMH= 410 nucleotides, while the SCR alignment which included RMH derived sequences from this study were trimmed to include the overlap region which was 167 nucleotides.** CIPRES Science Gateway (Miller et al., 2010) was used for substitution model determination using jModelTest2 (v2.1.6), and Bayesian phylogenetic tree construction using BEAST2 (v2.7.3). Convergence was assessed from the BEAST2 log output file using Tracer (v1.7.2) (Rambaut et al., 2018), which was inferred by effective sample size values of ≥200. The final phylogenetic tree was generated using TreeAnnotator (v.2.6.7) at 10 burnin, and edited using Figtree (v1.4.4) (Rambaut 2014 <http://tree.bio.ed.ac.uk/software/figtree/>) and Inkscape (v1.2) (Inkscape Project, 2020 Available from: <https://inkscape.org>). Nucleotide sequence identity matrix was generated using the trimmed alignment file via BioEdit with the “sequence identity matrix” function. For amino acid sequence identity matrix, the nucleotide alignment file was translated in the correct coding frame for an amino acid alignment. The final identities were converted from decimals to percentages. **For the nucleotide and amino acid sequence identity determination, selected sequences previously reported were included as indicated in Table 3.2 and Table 3.4.**

Chapter 3: Results

3.1 *Coronaviridae* detection and identification

3.1.1 Coronavirus screening in bat fecal samples

A total of 173 fecal samples were tested using the pan-coronavirus heminested assay for the partial amplification of the RdRp (polymerase) gene. The molecular detection was done by viral RNA extraction, followed cDNA synthesis. Detection using the pan-CoV primers was done, and the verification of positive samples was done using gel electrophoresis, with a positive result was indicated by observation of a 265 bp amplicon (see **Methodology 2.4.2** and **0**). Of the 173 samples tested, n=4 samples tested positive i.e., 2.31% all from pool DON008, which had 6 samples collected during the wet season from the horseshoe bat species. It should be noted no barcoding was performed to confirm the bat species. The positive samples were detected from fecal samples collected within the wet season from the horseshoe bat species. None of the samples collected within the dry season tested positive.

Table 3.1 summarizes the number of positives, with respect to the number of samples collected for each specific pool during the corresponding season of collection. Each pool represents a separate sampling event.

Table 3.1: Coronavirus screening of fecal samples using the Pancoronavirus hemi-nested RT-PCR assay

Season of Collection	Pool Sample ID	CoV positives/total tested	Proportion of positives (%)	Bat family ‡	Common Name*
Dry	DON005	0/14	0.00	Rhinolophidae	Horseshoe bat
	DON006	0/34	0.00	Rhinolophidae	Horseshoe bat
	DON007	0/51	0.00	Nycteridae	Common/Egyptian slit-faced bat
Wet	DON008	4/6	66.67	Rhinolophidae	Horseshoe bat
	DON009	0/27	0.00	Hipposideros	Commerson's leaf-nose bat
	DON010	0/41	0.00	Rhinolophidae	Horseshoe bat
Total CoV RNA detected: 4/173 (2.31%)					

* bat families suspected to be present in the environment

* all the bats from which fecal samples were collected are insectivorous bats.

3.1.1 Phylogenetic analyses

To determine the **relationship** of the pan-CoV positive sample sequences, bayesian phylogenetic analysis was performed. This was done by the determination of the substitution model using jModelTest2 with the use of a MSA file generated using sequences retrieved from GenBank (**Appendix Table 7.2**). Substitution model determination is crucial before the construction of a bayesian phylogenetic tree as it determines both the rate of substitution for either nucleotide or amino acid at a given site and also the distribution of the substitutions. For the determination of the substitution model, the MSA file in a phylip format was input on the CIPRES platform and the jModelTest2 tool was used for substitution model determination. The resulting output file included a summary of the scores of the various selection criterions of which the substitution model selected based on the Bayesian Criterion (BIC). The model subsequently was used in the creation of the BEAST2 in input file via BEAUti.

The findings of the coronavirus phylogenetic tree construction indicate the clustering of the positive CoV sequences with coronaviruses of the *Merbecovirus* genera as indicated in **Figure 3.1**. The sequences that cluster with those detected within this study include those previously detected in African bats. Samples DON008B003, DON008B006 and DON008B005 clustered with sequences previously reported in Zimbabwe in *Nycteris*, *Rhinolophus* and *Hipposideros* sp. bats. These include OM470177 BatCoV/*Rhinolophus*/MAG-1094/2021, OM469987 BatCoV/*Nycteris*/CHI-843/ZWE, OM469984 BatCoV/*Nycteris*/CHI-835/ZWE/2020, OM469985 BatCoV/CHI-851/ZWE/2020, OM470176 BatCoV/*Hipposideros*/MAG-1150/ZWE/202, and OM470177 BatCoV/*Rhinolophus*/MAG-1094/2021. The remaining sequence DON008B004 clustered with sequences previously reported in Zimbabwe OM469983 BatCoV/CHI-143/ZWE, and sequences previously identified in South Africa in *Miniopterus* bat, MK569443 BatCoV/*Miniopterus*/UP8489/LP/RSA/2017 and MK569432 BatCoV/*Miniopterus*/UP7530/GP/RSA/2017.

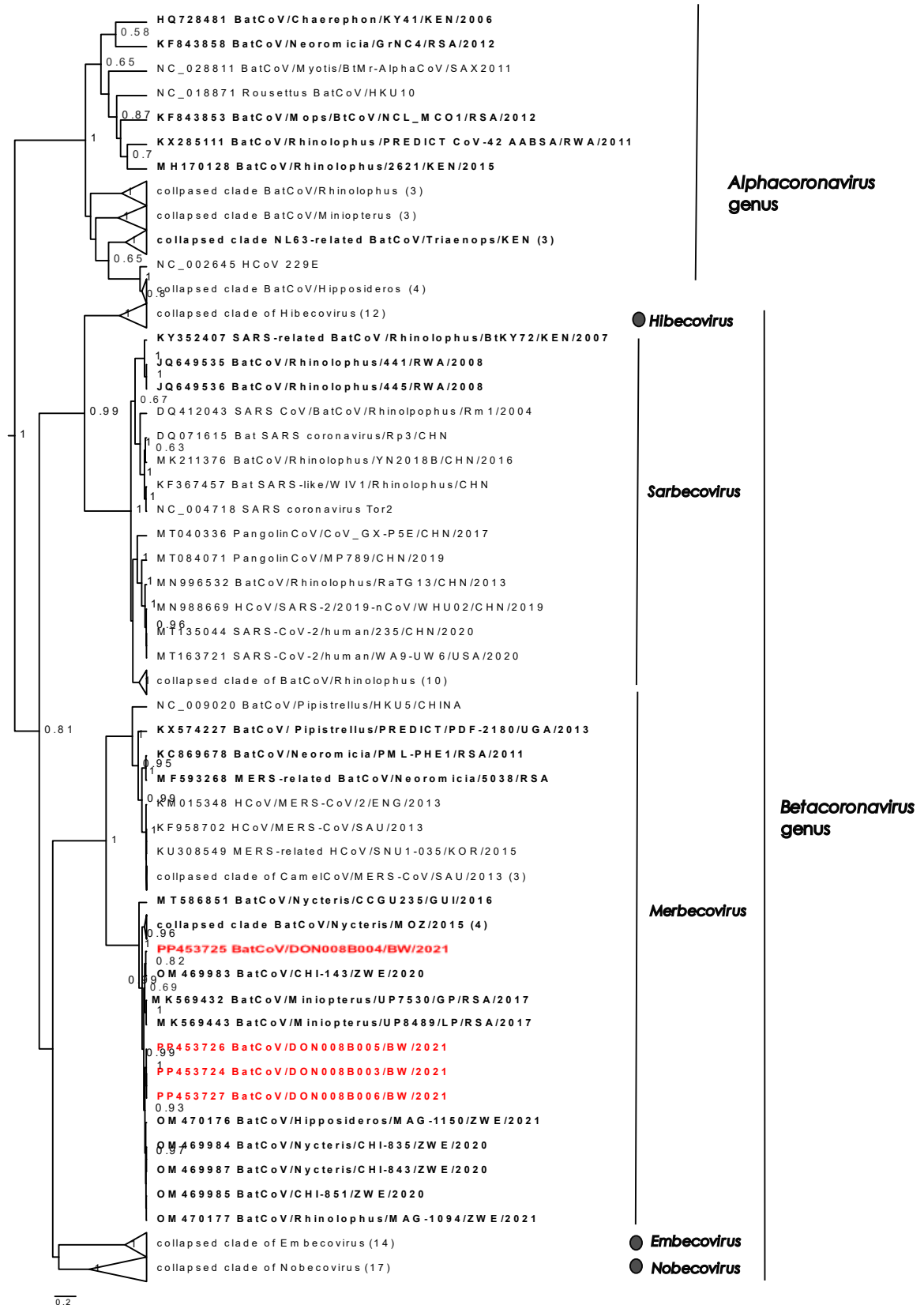


Figure 3.1: Phylogenetic analysis of coronaviruses detected in bats sampled at Gcwihaba caves.

Bayesian phylogenetic tree constructed with 113 representative sequences obtained from GenBank (21 AlphaCoV and 92 BetaCoV identified by the ascension number, host, and location). Sequence length, following to alignment, was trimmed to 207nt to include maximum number of sequences for the partial RdRp gene. Taxa in bold represents sequences identified in Africa, while sequences detected in this study are indicated in red and bold. For collapsed clades, numbers in brackets indicate the number of taxa found within the clade. Only Bayesian posterior values of ≥ 0.50 are shown at the internal nodes. The bayesian analysis was based on the TPMuf +I+G substitution model. The scale bar denotes the base substitution per site. The sequences for the AlphaCoV genus are the outgroup for phylogeny.

3.1.1 Nucleotide and amino acid sequence identities

Nucleotide (nt) and amino acid (aa) sequence similarity gives the degree of likeness between two sequences, while sequence identity indicates the number of character matches within two compared sequences. To perform nucleotide and amino acid sequence identity, multiple sequence alignment was carried which included Sanger-sequenced positive samples, and reference sequences obtained from NCBI. The MSA was done using the ClustalW function of BioEdit. Once the alignment output was obtained, the sequence identity matrix function of BioEdit was used to determine the nt sequence identity. For the aa sequence identity, the MSA output file was toggled to give the aa sequences, followed by the sequence identity matrix function.

Table 3.2: Sequences included for nucleotide and amino acid sequence identity comparison

Genus	CoV subgenus	Accession ID	Name	Host	Reference
Betacoronavirus	<i>Nobecovirus</i>	HM211099	Bat coronavirus HKU9-5-2	bat	(Lau et al., 2010)
		NC030886	Rousettus bat coronavirus GCCDC1 356	<i>Rousettus leschenaulti</i> (bat)	
	<i>Embecovirus</i>	KP112152	Human coronavirus HKU1	<i>Homo sapiens</i> (human)	(Sipulwa et al., 2016)
		MN026164	Human coronavirus OC43 isolate OC43_KLF_01_2018	<i>Homo sapiens</i> (human)	(Kamau et al., 2019)
	<i>Merbecovirus</i>	OM469983	Bat betacoronavirus isolate CHI-143	Bat sp.	(Chidoti et al., 2022)
		OM469987	Bat alphacoronavirus isolate CHI-843*	<i>Nycteris</i> sp. (bat)	
		OM469984	BatCoV/Nycteris/CHI-835/ZWE/2020	<i>Nycteris</i> sp. (bat)	
		OM469985	BatCoV/CHI-851/ZWE/2020	Bat sp.	
		OM470176	BatCoV/Hipposideros/M AG-1150/ZWE/2021	<i>Hipposideros</i> sp. (bat)	
		OM470177	BatCoV/Rhinolophus/M AG-1094/2021	<i>Rhinolophus</i> (bat)	
		MK569432	Betacoronavirus sp. strain BtCoV/Miniopterus/UP7 530/GP/RSA/2017	<i>Miniopterus natalensis</i> (bat)	Nkambule, Geldenhuys & Markotter, 2019)†
		MK569453	Betacoronavirus sp. strain BtCoV/Nycteris/UP6323 /LP/RSA/2016	<i>Nycteris thebaica</i> (bat)	
		EF203066	Bat coronavirus HKU2 strain HKU2/HK/298/2006	<i>Rhinolophus sinicus</i> (bat)	(Lau et al., 2007)

		MG923468	MERS-CoV camel/Ethiopia/AAU-EPHI-HKU4458/2017	<i>Camelus dromedarius</i> (dromedary camel)	(Chu et al., 2018)
		KJ473821	BtVs-BetaCoV/SC2013	<i>Vespertilio superans</i> (bat)	(Yang et al., 2014)
		KX574227	Bat coronavirus isolate PREDICT/PDF-2180	<i>Pipistrellus hesperidus</i> (bat) cf.	(Anthony et al., 2017)
		KC869678	Coronavirus Neoromicia/PML-PHE1/RSA/2011	<i>Neoromicia capensis</i> (bat)	(Ithete et al., 2013)
		NC_009019	Tylonycteris bat coronavirus HKU4	<i>Tylonycteris pachypus</i> (bat)	(Woo et al., 2007)
	<i>Sarbecovirus</i>	KY352407	Severe acute respiratory syndrome-related coronavirus strain BtKY72	<i>Rhinolophus</i> sp. (bat)	(Tao & Tong, 2019)
		JQ649536	Rhinolophus bat coronavirus/Rwanda/445/2008 isolate 445	<i>Rhinolophus clivosus</i> (bat)	(Markotter et al., 2019)
		MH170126	Bat coronavirus isolate BatCoV2368/KEN/Meru	<i>Hipposideros caffer</i> (bat)	Waruhiu et al., 2017
		MG000872	Bat SARS-like coronavirus isolate BtCov-Zim035Mag	<i>Hipposideros</i> sp. (bat)	(Bourgarel et al., 2018)
Alphacoronavirus	<i>Decacovirus</i>	NC018871	Rousettus bat coronavirus HKU10	bat	(Lau et al., 2012)
	<i>Myotacovirus</i>	NC028811	BtMr-AlphaCoV/SAX2011	<i>Myotis ricketti</i> (bat)	(Wu et al., 2016)
		KF843858	Alphacoronavirus BtCoV/GrNC4/Neo_cap/RSA/2012	<i>Neoromicia</i> cf. <i>capensis</i> (bat)	Ithete et al., 2013) †
		HQ728481	Chaerephon bat coronavirus/Kenya/KY41/2006	<i>Chaerephon pumilus</i> (bat)	(Tong et al., 2009)

*Entry listed under Alphacoronavirus in GenBank but cluster within Betacoronaviruses in this study

† Unpublished

Nucleotide and amino acid sequence identity matrices for the detected CoV assay are summarized below in **Table 3.3**. Blanks cells are where a particular sequence has been excluded within the sequence identity comparison.

The detected BatCoV sequences from this study had a nucleotide sequence identity range 94.6 – 100.0%, and amino acid sequence identity between 95.6 – 100.0% with select betacoronaviruses. The select sequences include OM469983 BatCoV/CHI-143/ZWE, OM469987 BatCoV/Nycteris/CHI-843/ZWE, OM469984 BatCoV/Nycteris/CHI-835/ZWE/2020, OM469985 BatCoV/CHI-851/ZWE/2020, OM470176 BatCoV/Hipposideros/MAG-1150/ZWE/2021, OM470177 BatCoV/Rhinolophus/MAG-1094/2021, MK569432 BatCoV/Miniopterus/UP7530/GP/RSA/2017, MK569453 BatCoV/Miniopterus/UP8489/LP/RSA/2017). Three sequences, DON008B003, DON008B005

and DON008B006 have 100 pairwise nucleotide and amino acid sequence identity when compared to each other. The remaining sequence, DON008B004, has a nucleotide sequence identity of 96.6 and 97.1 amino acid sequence identity when compared to the other three sequences detected. **Notably, the assay derived sequences also show a close genetic relationship with the MERS-related coronaviruses previously reported in bats, KJ473821.1 BtVs-BetaCoV/SC2013, KX574227.1 Bat coronavirus isolate PREDICT/PDF-2180, KC869678.4 Coronavirus Neoromicia/PML-PHE1/RSA/2011 and a MERS-related coronaviruses detected in a dromedary camel, MG923468.1 MERS-CoV camel/Ethiopia/AAU-EPHI-HKU4458/2017. The nucleotide and amino acid sequence identity of the assay derived sequences was 69.00-71.00% and 79.70-85.50% respectively.**

To elaborate the sequence identity information indicated in **Table 3.3**, molecular evolutionary divergence is given in **Appendix Table 7.3** for further emphasis of the similarity between the sequences identified within this study and classified coronavirus sequences indicated in Table 3.2. The sequences that had greater sequence identity, subsequently showed lesser evolutionary divergence.

Table 3.3: Pairwise percentage (%) sequence identity based on alignment of partial RdRp gene.

Selected coronaviruses (alpha- and betaCoV) and sequences identified in this study (highlighted in red and bold) were used in the calculation of identity. The nucleotide identity (upper right of the diagonal shown by grey cells) is based on a 207-nucleotide alignment, while the amino acid identity (lower left of the diagonal) is based on a 69 amino acid alignment. Percent identities highlighted in bold indicate scores >95.00 between selected CoV sequences and sequences obtained in this study.

Seq->	DON008B00 3	DON008B00 4	DON008B00 5	DON008B00 6	HM211099	NC_030886	KP112152	MN026164	OM469983	OM469984	OM469985	OM469987	OM470176	OM470177	MK569432	MK569443	EF203066	MG923468	KJ473821.	KX574227	KC869678	NC_009019	KY352407	JQ649536	MH170126	MG000872	NC_018871	NC_028811	KF843858_1	HQ728481
BatCoV/Rhinolophus/DON008B03/BW/2021		96.60	100.00	100.00	61.80	55.50	65.70	60.80	97.50	97.50	97.50	97.50	96.10	97.10	95.60	96.10	52.10	70.50	69.00	71.00	71.90	75.30	62.30	63.20	59.40	60.80	55.00	50.20	55.50	59.40
BatCoV/Rhinolophus/DON008B04/BW/2021	97.10		96.60	96.60	61.30	54.50	65.20	61.80	99.00	96.10	96.10	96.10	94.60	95.60	97.10	97.50	52.60	71.40	70.50	71.40	72.40	73.90	60.80	61.80	61.30	61.30	55.50	48.70	54.10	59.40
BatCoV/Rhinolophus/DON008B05/BW/2021	100.00	97.10		100.00	61.80	55.50	65.70	60.80	97.50	97.50	97.50	97.50	96.10	97.10	95.60	96.10	52.10	70.50	69.00	71.00	71.90	75.30	62.30	63.20	59.40	60.80	55.00	50.20	55.50	59.40
BatCoV/Rhinolophus/DON008B06/BW/2021	100.00	97.10	100.00		61.80	55.50	65.70	60.80	97.50	97.50	97.50	97.50	96.10	97.10	95.60	96.10	52.10	70.50	69.00	71.00	71.90	75.30	62.30	63.20	59.40	60.80	55.00	50.20	55.50	59.40
HM211099_1 BatCoV/ HKU9-5-2	53.60	53.60	53.60	53.60		74.80	64.20	64.80	60.80	60.80	60.80	60.80	59.90	60.30	59.40	59.40	62.30	59.40	57.90	57.90	58.90	57.00	67.60	66.10	73.90	58.90	56.50	62.30	60.80	63.70
NC_030886_1 Roussetus bat coronavirus GCCDC1 356	52.10	52.10	52.10	52.10	86.90		63.20	61.30	55.00	56.00	56.00	56.00	55.50	55.50	55.50	55.50	54.50	55.50	54.10	54.50	55.50	55.00	60.30	59.90	58.40	57.40	55.00	61.30	57.90	58.40
KP112152_1	57.90	59.40	57.90	57.90	55.00	56.50		78.70	66.10	64.20	64.20	64.20	63.70	63.70	64.20	64.20	57.40	59.40	62.30	60.80	61.30	64.20	62.30	62.30	64.20	66.50	55.50	58.40	58.40	63.70
HCoV/HKU1/KENYA/008/2009	90.40	90.40	90.40	90.40	00.50			70.10	20.20	20.20	20.20	20.20	70.20	70.20	70.20	70.20	40.90	90.30	80.30	80.30	70.30	70.30	80.30	30.70	60.70	60.00	40.40	90.40	90.70	70.40
MN026164_1	59.40	60.40	59.40	59.40	59.40	60.80	85.50		62.30	59.90	59.90	59.90	59.40	59.40	61.80	61.80	55.00	57.40	59.90	56.40	59.40	62.30	58.90	58.40	61.30	63.20	52.10	56.00	57.90	57.40
HCoV/OC43/KLF/01/2018/KENYA	40.80	40.80	40.40	40.40	40.80	80.50			30.90	90.90	90.90	90.40	40.80	40.80	80.80	80.80	00.40	40.90	50.40	70.40	40.30	90.30	90.40	40.80	60.20	10.00	00.00	00.90	90.40	59.40
OM469983.1 BatCoV/CHI-143/ZIMBABWE	97.10	100.00	97.10	97.10	53.60	52.10	59.40	60.80		97.10	97.10	97.10	95.60	96.10	98.00	98.50	52.60	71.90	70.00	72.90	74.90	61.30	62.30	60.80	61.30	56.80	48.70	54.10	59.40	40.40
OM469984 BatCoV/Nycteris/CHI-835/ZWE/2020	98.50	98.50	98.50	98.50	53.60	52.10	57.90	59.40	98.50		100.00	100.00	98.50	99.50	97.10	97.50	50.20	71.40	69.40	71.40	71.40	72.90	61.30	62.30	59.40	62.30	55.00	49.70	54.10	58.40
OM469985 BatCoV/CHI-851/ZWE/2020	98.50	98.50	98.50	98.50	53.60	52.10	57.90	59.40	98.50	100.00		100.00	98.50	99.50	97.10	97.50	50.20	71.40	69.40	71.40	71.40	72.90	61.30	62.30	59.40	62.30	55.00	49.70	54.10	58.40
OM469987 BatCoV/Nycteris/CHI-843/ZWE	98.50	98.50	98.50	98.50	53.60	52.10	57.90	59.40	98.50	100.00	100.00		98.50	99.50	97.10	97.50	50.20	71.40	69.40	71.40	71.40	72.90	61.30	62.30	59.40	62.30	55.00	49.70	54.10	58.40
OM470176																														
BatCoV/Hipposideros/MAG-1150/ZWE/2021	95.60	95.60	95.60	95.60	52.10	50.70	56.50	57.90	95.60	97.10	97.10	97.10		98.00	95.60	96.10	50.70	71.00	67.00	71.00	71.00	72.90	60.30	61.30	58.40	61.30	55.50	49.20	53.10	57.00
OM470177																														
BatCoV/Rhinolophus/MAG-1094/2021	97.10	97.10	97.10	97.10	52.10	50.70	56.50	57.90	97.10	98.50	98.50	98.50	95.60		96.60	97.10	49.70	71.00	68.50	71.00	71.00	72.90	60.80	61.30	58.90	61.30	54.50	49.20	53.60	57.90
MK569432.1																														
BatCoV/Miniopterus/UP7530/GP/R SA/2017	97.10	100.00	97.10	97.10	53.60	52.10	59.40	60.80	100.00	98.50	98.50	98.50	95.60	97.10		99.50	52.60	71.90	70.50	71.90	71.90	73.90	60.30	61.30	59.90	61.30	54.50	48.70	53.10	57.40
MK569443.1																														
BatCoV/Miniopterus/UP8489/LP/R SA/2017	97.10	100.00	97.10	97.10	53.60	52.10	59.40	60.80	100.00	98.50	98.50	98.50	95.60	97.10	100.00		52.60	72.40	71.00	72.40	72.40	74.90	60.30	61.30	59.90	62.30	55.00	48.70	53.60	57.90
EF203066.1 Bat coronavirus HKU2 strain HKU2/HK/298/2006	49.20	49.20	49.20	49.20	59.40	59.40	46.30	52.10	49.20	49.20	49.20	49.20	49.20	47.80	49.20	49.20		53.60	54.10	54.10	54.10	55.50	62.30	62.30	58.40	55.50	64.70	73.40	66.60	70.00

MG923468.1 MERS-CoV camel/Ethiopia/AAU-EPHI-HKU4458/2017	85.	84.	85.	85.	53.	50.	60.	62.	84.	84.	84.	84.	81.	82.	84.	84.	47.		85.	89.	92.	74.	60.	62.	60.	66.	55.	56.	57.	57.
KJ473821.1 BtVs-BetaCoV/SC2013	50	00	50	50	60	70	80	30	00	00	00	00	10	60	00	00	80		00	30	20	30	80	80	80	60	50	50	40	90
KX574227.1 Bat coronavirus isolate PREDICT/PDF-2180	81.	79.	81.	81.	52.	49.	63.	63.	79.	79.	79.	79.	76.	78.	79.	79.	43.	89.		85.	83.	77.	59.	59.	63.	65.	51.	57.	54.	55.
KC869678.4 Coronavirus Neoromicia/PML-PHE1/RSA/2011	10	70	10	10	10	20	70	70	70	70	70	70	80	20	70	70	40	80		50	50	70	40	90	70	70	20	00	50	50
NC_009019.1 Tylonycteris bat coronavirus HKU4	82.	81.	82.	82.	53.	50.	60.	62.	81.	81.	81.	81.	78.	79.	81.	81.	47.	95.	89.		90.	74.	62.	64.	61.	64.	53.	57.	60.	56.
KY352407_1 SARS-related BatCoV/Rhinolophus/KENYA/BtKY72	60	10	60	60	60	70	80	30	10	10	10	10	20	70	10	10	80	60	80		80	80	30	70	30	70	60	40	80	50
JQ649536_2 BatCoV/Rhinolophus/Rwanda/445/2008	85.	84.	85.	85.	53.	50.	60.	62.	84.	84.	84.	84.	81.	82.	84.	84.	47.	98.	89.	97.		76.	59.	61.	61.	65.	55.	59.	58.	57.
MH170126_1 BatCoV/ Hipposideros/2368/KENYA	50	00	50	50	60	70	80	30	00	00	00	00	10	60	00	00	80	50	80	10		30	90	80	30	70	50	40	40	40
MG000872_1 Bat SARS-like/BtCov-Zim035Mag	79.	78.	79.	79.	50.	49.	60.	62.	78.	78.	78.	78.	76.	76.	78.	78.	46.	86.	84.	84.	86.		60.	61.	59.	62.	53.	59.	57.	57.
NC_018871_1 Roussettus bat coronavirus HKU10	70	20	70	70	70	20	80	30	20	20	20	20	80	80	20	20	30	90	00	00	90		80	30	90	80	10	40	40	90
NC_028811_1 BatCoV/Myotis/BtMr-AlphaCoV/SAX2011	62.	63.	62.	62.	65.	66.	65.	66.	63.	63.	63.	63.	62.	62.	63.	63.	56.	62.	62.	62.	62.	59.		97.	66.	64.	54.	59.	61.	60.
KF843858_1 BatCoV/Neoromicia/GrNC4/Neo_cap/RSA/2012	30	70	30	30	20	60	20	60	70	70	70	70	30	30	70	70	50	30	30	30	30	40		10	10	70	10	40	80	30
HQ728481_1 BatCoV/Chaerephon/Kenya/KY41/2006	62.	63.	62.	62.	65.	66.	65.	66.	63.	63.	63.	63.	62.	62.	63.	63.	56.	62.	62.	62.	62.	59.	100		65.	63.	54.	58.	62.	60.
	30	70	30	30	20	60	20	60	70	70	70	70	30	30	70	70	50	30	30	30	30	40	.00		70	70	50	90	30	30
	65.	66.	65.	65.	68.	63.	66.	71.	66.	66.	66.	66.	63.	65.	66.	66.	56.	66.	68.	66.	66.	68.	69.	69.		81.	54.	56.	56.	64.
	20	60	20	20	10	70	60	00	60	60	60	60	70	20	60	60	50	60	10	60	60	10	50	50		60	10	50	50	70
	68.	69.	68.	68.	62.	62.	68.	69.	69.	69.	69.	69.	66.	68.	69.	69.	52.	68.	71.	68.	68.	68.	71.	71.	86.		55.	56.	60.	62.
	10	50	10	10	30	30	10	50	50	50	50	50	60	10	50	50	10	10	00	10	10	10	00	00	90		00	50	30	80
	56.	56.	56.	56.	55.	53.	47.	50.	56.	56.	56.	56.	56.	55.	56.	56.	75.	53.	46.	52.	52.	49.	52.	52.	55.	53.		67.	71.	67.
	50	50	50	50	00	60	80	70	50	50	50	50	50	00	50	50	30	60	30	10	10	20	10	10	00	60		10	40	60
	49.	49.	49.	49.	60.	63.	52.	60.	49.	49.	49.	49.	49.	47.	49.	49.	81.	53.	49.	53.	53.	52.	60.	60.	59.	56.	73.		74.	71.
	20	20	20	20	80	70	10	80	20	20	20	20	20	80	20	20	10	60	20	60	60	10	80	80	40	50	90		80	90
	49.	49.	49.	49.	59.	60.	52.	56.	49.	49.	49.	49.	49.	47.	49.	49.	69.	52.	46.	52.	52.	49.	60.	60.	56.	56.	71.	82.		74.
	20	20	20	20	40	80	10	50	20	20	20	20	20	80	20	20	50	10	30	10	10	20	80	80	50	50	00	60		30
	53.	53.	53.	53.	57.	59.	56.	56.	53.	53.	53.	53.	53.	52.	53.	53.	72.	55.	49.	55.	55.	55.	60.	60.	59.	59.	75.	79.	88.	
	60	60	60	60	90	40	50	50	60	60	60	60	60	10	60	60	40	00	20	00	00	00	80	80	40	40	30	70	40	

3.2 Paramyxoviridae detection and identification

3.2.1 Paramyxovirus screening in bat fecal samples

To determine the presence of paramyxoviruses within the fecal samples (N=171), two RT-PCR assays were used. For samples where the RMH assay was inconclusive, the SCR assay was used. The screening positivity for the PMV viral RNA was found to be 30.41% (n=52 positive samples). The full outline of positives per sample ID is detailed in (**Appendix Table 7.1**). The viral nucleic acid positivity for the respective assays is summarized in **Table 3.4**.

The dry season had a higher proportion of positives in the samples tested (41 positive samples), with majority of these positives (38 overall) attributed to Nycteridae bats, which were only detected in the dry season and account for most of the positive samples. In contrast, the wet season had a lower number of positives (11 samples). This difference underscores the significant contribution of Nycteridae bats to the overall positivity rate, accounting for nearly 74% out of the 51 samples tested.

Table 3.4: Paramyxovirus screening of bat fecal samples

Season of collection	Pool Sample ID	RMH positives/total tested	Proportion of positives (%)	SCR positives/total of samples retested	Proportion of positives (%)	Bat Family †	Common Name*
Dry	DON005	0/14	0	N/A	NA	Rhinolophidae	Horseshoe bat
	DON006	0/32	0	3/6	50	Rhinolophidae	Horseshoe bat
	DON007	23/51	45.1	15/15	100	Nycteridae	Common/Egyptian slit-faced bat
Wet	DON008	0/6	0	N/A	NA	Rhinolophidae	Horseshoe bat
	DON009	4/27	14.8	3/4	75	Hipposideridae	Commerson's leaf-nose bat
	DON010	3/41	7.32	1/3	33.3	Rhinolophidae	Horseshoe bat
Total PMV RNA detected : 52/171 (30.41%)							

† bat families suspected to be present in the environment

N/A: not applicable i.e., assay not performed

3.2.1 RMH RT-PCR phylogenetic and sequence identity analyses

Paramyxovirus phylogenetic analysis of the partial L gene is shown in **Figure 3.2**: Phylogenetic analysis of Paramyxoviruses detected in bats sampled at Gcwihaba caves using the RMH assay below based on the 410-bp trimmed MSA from the heminested RMH assay

and included animal and human partial sequences retrieved from NCBI (See **Appendix Table 7.4**).

The phylogenetic tree in **Figure 3.2** shows that all sequences associated with the Nycteridae bats group together within the putative Jeilongvirus viruses clade. Additionally, these sequences cluster with previously reported *Nycteris* bat-associated sequence, KC578581_BatPV/*Nycteris*/RSA-855/2010. In comparison, sequences associated with the Rhinolophidae and Hipposideridae bats cluster within the Jeilongvirus related viruses clade. Sequences formerly reported in Africa that cluster with the RMH RT-PCR derived sequences include those from Nigeria (KC538903 BatPV/NIG955/NIG/2010), Cameroon (KC578678 BatPV/*Rhinolophus*/CAM-45/2010 and KC578681 BatPV/*Hipposideros*/CAM-88/2010), Ghana (HQ660153 BatPV/*Hipposideros*/GH19-140/GHA/2009), Democratic Republic of Congo (KC578675 BatPV/*Hipposideros*/DRC-328/2011), and South Africa (MK967695 BatPV/*Hipposideros*/UP450/RWA/2008). The representative sequences mentioned are also associated with the *Rhinolophus* and *Hipposideros* bats, suggesting possible virus sharing within the bat genera. Furthermore, *Nycteris* derived sequences show distant genetic relationship with other genus derived sequences i.e., *Hipposideros* and *Rhinolophus* bat derived sequences.

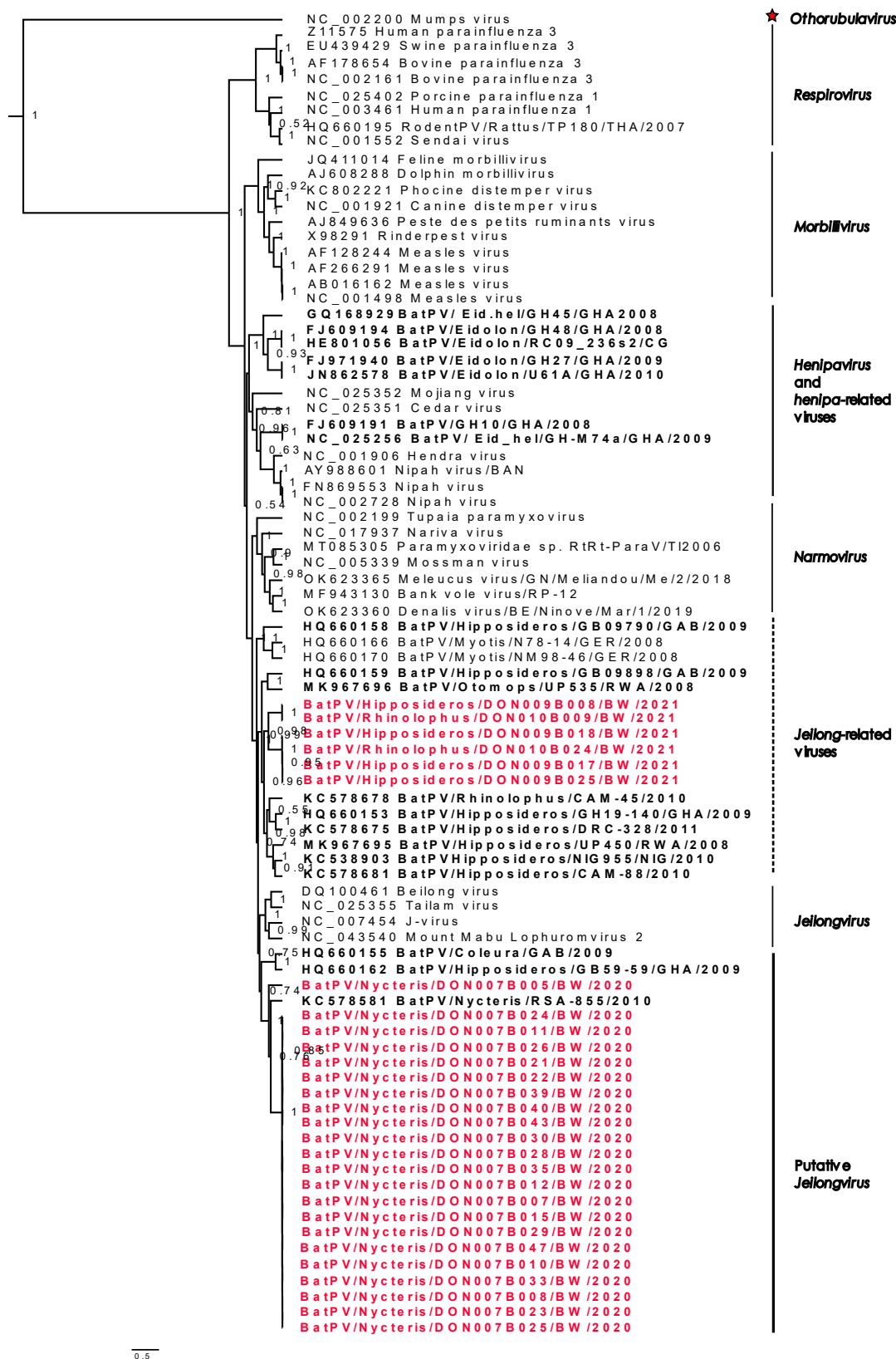


Figure 3.2: Phylogenetic analysis of Paramyxoviruses detected in bats sampled at Gcwihaba caves using the RMH assay. Bayesian phylogenetic tree constructed with 57 representative sequences obtained from GenBank identified by the ascension number, host, and location. Sequence length, following alignment, was trimmed to 410 nt to include maximum number of sequences for the partial polymerase (L) gene. Taxa in bold represents sequences identified in Africa, while sequences detected in this study are indicated in red and bold. For collapsed clades, numbers in brackets indicate the number of taxa found within the clade. Only Bayesian posterior values of ≥ 0.50 are shown at the internal nodes. The bayesian analysis was based on the TIM3 +I+G substitution model. The scale bar denotes the base substitution per site. Mumps virus was selected as an outgroup for phylogeny.

Sequence identity for the **PMV** was performed as described in **Methodology**, section 2.9. The table below (**Table 3.5**) gives a summary of selected sequences included in the percentage sequence identity identification.

Table 3.5: Specific **PMV sequences included for identity determination**

Genus	GenBank Accession	Virus Identity	Host	Reference
Jeilongvirus	NC_007454	J-virus	House mouse (<i>Mus musculus</i>)	(Jack et al., 2005)
Jeilongvirus	NC_025355	Tailam virus	Sikkim rat (<i>Rattus andamanensis</i>)	(Woo et al., 2011)
Jeilongvirus	NC_043540	Mount Mabu Lophuromys virus 2	Brush-furred mouse (<i>Lophuromys machangui</i>)	(Vanmechelen et al., 2017) [‡]
Jeilongvirus	DQ100461	Beilong virus	Rodent (<i>Rattus norvegicus</i>)	(Li et al., 2006)
Jeilongvirus-related	KC538903	BatPV/Hipposideros/NIG955/NIG/2010	Bat (<i>Hipposideros</i> sp.)	(Mortlock, 2013)
Jeilongvirus-related	HQ660153	BatPV/Hipposideros/GH19-140/GHA/2009	Bat (<i>Hipposideros ruber</i>)	(Drexler et al., 2012)
Jeilongvirus-related	HQ660155	BatPV/Coleura/GAB/2009	Bat (<i>Coleura afra</i>)	(Drexler et al., 2012)
Jeilongvirus-related	HQ660158	BatPV/Hipposideros/GB09790/GAB/2009	Bat (<i>Hipposideros caffer</i>)	(Drexler et al., 2012)
Jeilongvirus-related	HQ660159	BatPV/Hipposideros/GB09898/GAB/2009	Bat (<i>Hipposideros gigas</i>)	(Drexler et al., 2012)
Jeilongvirus-related	HQ660162	BatPV/Hipposideros/GB59-59/GHA/2009	Bat (<i>Hipposideros abae</i>)	(Drexler et al., 2012)
Jeilongvirus-related	HQ660166	BatPV/Myotis/N78-14/GER/2008	Bat (<i>Myotis myotis</i>)	(Drexler et al., 2012)
Jeilongvirus-related	HQ660170	BatPV/Myotis/NM98-46/GER/2008	Bat (<i>Myotis bechsteinii</i>)	(Drexler et al., 2012)
Jeilongvirus-related	KC578581	BatPV/Nycteris/RSA-855/2010	Bat (<i>Nycteris thebaica</i>)	(Mortlock, 2013)
Jeilongvirus-related	KC578675	BatPV/Hipposideros/DRC-328/2011	Bat (<i>Hipposideros fuliginosus</i>)	(Mortlock, 2013)
Jeilongvirus-related	KC578678	BatPV/Rhinolophus/CAM-45/2010	Bat (<i>Rhinolophus</i> sp.)	(Mortlock, 2013)
Jeilongvirus-related	KC578681	BatPV/Hipposideros/CAM-88/2010	Bat (<i>Hipposideros</i> sp.)	(Mortlock, 2013)
Jeilongvirus-related	MK967695	BatPV/Hipposideros/UP450/RWA/2008	Bat (<i>Hipposideros ruber</i>)	(Mortlock et al., 2019) [‡]
Jeilongvirus-related	MK967696	BatPV/Otomops/UP535/RWA/2008	Bat (<i>Otomops martiensseni</i>)	(Mortlock et al., 2019) [‡]

* unpublished

[‡] used in SCR analysis sequence identity analysis only

Pairwise amino acid (aa) sequence identity was done to compare all sequences detected using the RMH assay, **applying** a cut-off of 94.0% per criterion detailed by Mortlock et al., (2021) **to demarcate unique sequences and sequences being detected at one time. It is important to note that this method serves as a rough indication due to the limited sequence information available from surveillance studies.** Sequences, DON007B007, DON009B017,

DON010B009, and **DON007B005** were selected as representative sequences and used in pairwise sequence identity with previously characterized sequences indicated in **Table 3.5**. Full information on aa identity of detected sequences is shown in **Appendix Table 7.5**.

The pairwise nucleotide sequence (nt) identity ranged from 65.1 to 73.6%, while the aa identity ranged from 63.2 to 77.9%. Nucleotide sequence identity ranges for the four chosen sequences were found to be 65.8-73.1% (DON009B017), 62.1-72.9% (DON007B005), 65.6-76.5% (DON007B007) and 64.3-71.7 (DON010B009) when compared to previously characterized viral sequences. The corresponding aa sequence identity ranges were 69.8-77.2%, 58.0-79.4%, 68.3-88.2% and 66.9-78.6% respectively. Based on aa sequence identity, DON009B017 exhibited a close relationship with a previously reported Gabon sequence (HQ660159 BatPV/ *Hipposideros*/GB09898/GAB/2009), showing 77.2% identity. In the study, DON009B017 was associated with a *Hipposideros* spp. bat. Similarly, DON010B009, associated with *Rhinolophus* spp. bat, showed a close relationship with KC578678 BatPV/*Rhinolophus*/CAM-45/2010, previously reported in Cameroon, at 78.6% aa identity. The sequences DON007B005 and DON007B007, both associated with *Nycteris* spp. bats, exhibited a close relationship with a sequence previously reported in South Africa (KC578581 BatPV/*Nycteris*/RSA-855/2010), at aa identities of 79.4% and 88.2%, respectively. To supplement the relationship between detected sequences with previously characterized sequences, the genetic distance is shown in **Appendix Table 7.6**.

Table 3.6: Pairwise percent (%) sequence identity for the partial polymerase (L) gene using selected **PMV sequences**

Selected **PMV** sequences and sequences identified in this study (highlighted in red and bold) were used in the calculation of identity. The nucleotide identity (upper right of the diagonal) is based on a 410-nucleotide alignment, while the amino acid identity (lower left of the diagonal) is based on a 136 amino acid alignment. Pairwise sequence identity scores of ≥ 70.0 are indicated in bold for comparison between study sequences and literature sequences. For the sample derived sequences for this study, only one representative was included in the table with subsequent sequences within the cluster highlighted below the table.

Seq->	DON009B017 ¹	DON007B005 ⁺	DON007B007 ²	DON010B009 ³	DQ100461	HQ660153	HQ660155	HQ660158	HQ660159	HQ660162	HQ660166	HQ660170	KC538903	KC578581	KC578675	KC578678	KC578681	MK967695	MK967696	NC_007454	NC_025355	NC_043540
BatPV/Hipposideros/DON009B017/BW/2021 ¹	ID	66.8	68.0	73.6	65.8	70.9	68.2	69.2	70.0	68.7	69.2	67.0	72.1	69.7	71.2	70.9	72.6	73.1	68.2	68.5	69.2	68.2
BatPV/Nycteris/DON007B005/BW/2020 ⁺	67.6		70.4	65.1	62.1	65.8	64.1	64.1	63.4	63.9	66.5	62.4	64.8	72.9	66.0	63.6	67.8	67.0	65.1	63.6	66.8	66.0
BatPV/Nycteris/DON007B007/BW/2020 ²	72.7	79.4		65.8	66.3	72.1	67.3	67.0	68.2	68.7	69.0	66.3	70.2	76.5	70.9	69.0	70.7	70.7	68.5	65.6	70.0	68.2
BatPV/Rhinolophus/DON010B009/BW/2021 ³	77.9	63.2	71.3		67.3	69.5	66.0	64.3	71.4	66.0	66.0	66.8	71.7	68.2	69.2	69.7	71.2	68.7	70.0	65.1	68.7	66.0
DQ100461 Beilong virus	72.7	65.4	72.0	69.1		66.3	65.1	65.1	65.1	64.1	65.6	64.3	64.3	68.2	67.8	63.6	65.8	67.5	69.5	70.7	74.6	66.5
HQ660153 BatPV/Hipposideros/GH19-140/GHA/2009	75.7	61.7	73.5	75.0	72.0		69.2	71.7	71.4	70.9	71.9	68.7	76.3	70.9	82.9	73.4	74.6	76.3	73.9	67.3	65.6	67.0
HQ660155 BatPV/Coleura/GAB/2009	70.5	63.9	72.7	69.8	69.8	77.2		64.6	68.7	81.7	69.0	66.0	72.1	70.7	68.0	66.3	68.7	70.4	67.3	64.1	65.6	63.4
HQ660158 BatPV/Hipposideros/GB09790/GAB/2009	69.8	60.2	72.0	66.9	71.3	75.0	69.8		66.8	66.0	69.5	70.7	70.7	68.0	69.5	65.3	67.8	69.7	66.3	65.8	66.8	65.8
HQ660159 BatPV/Hipposideros/GB09898/GAB/2009	77.2	63.9	75.0	78.6	72.7	75.0	73.5	72.0		69.7	69.2	66.3	73.4	67.3	70.9	71.4	69.7	70.4	71.7	67.5	65.6	66.5
HQ660162 BatPV/Hipposideros/GB59-59/GHA/2009	69.8	62.5	70.5	66.9	69.8	75.0	93.3	69.8	72.0		70.2	67.5	71.4	69.0	70.9	68.5	68.5	66.5	70.2	61.7	62.6	64.3
HQ660166 BatPV/ Myotis/N78-14/GER/2008	74.2	66.9	72.0	70.5	71.3	71.3	72.7	75.0	75.0	72.7		76.5	70.9	68.5	65.1	66.0	69.0	69.2	67.5	66.5	66.5	64.6
HQ660170 BatPV/ Myotis/NM98-46/GER/2008	72.0	63.2	68.3	69.1	69.8	71.3	71.3	77.9	72.7	72.0	89.7		69.0	67.8	65.1	66.8	67.0	68.5	65.1	64.8	62.6	64.3
KC538903 BatPV/NIG955/NIG/2010	76.4	62.5	73.5	75.0	73.5	83.0	77.2	73.5	75.7	77.2	73.5	72.7		70.0	76.0	73.4	80.9	80.4	74.1	63.4	67.0	66.0
KC578581 BatPV/Nycteris/RSA-855/2010	73.5	79.4	88.2	68.3	72.7	72.0	72.0	67.6	71.3	70.5	74.2	69.8	69.8		70.9	67.0	69.7	70.9	69.2	64.3	69.0	67.8
KC578675 BatPV/Hipposideros/DRC-328/2011	75.7	63.2	75.0	73.5	74.2	97.0	75.7	75.0	72.7	75.0	70.5	70.5	83.8	72.7		74.1	75.1	74.6	73.1	65.8	65.8	67.3
KC578678 BatPV/Rhinolophus/CAM-45/2010	75.7	58.0	72.7	78.6	69.8	80.8	70.5	69.1	77.2	69.1	68.3	69.8	79.4	68.3	79.4		73.4	72.1	68.5	65.3	65.8	65.8
KC578681 BatPV/Hipposideros/CAM-88/2010	75.7	63.2	75.7	76.4	74.2	83.8	73.5	72.7	75.7	73.5	71.3	71.3	90.4	72.0	83.8	83.0		78.2	72.6	65.1	70.0	68.2

Seq->	DON009B017 ¹	DON007B005 [†]	DON007B007 ²	DON010B009 ³	DQ100461	HQ660153	HQ660155	HQ660158	HQ660159	HQ660162	HQ660166	HQ660170	KC538903	KC578581	KC578675	KC578678	KC578681	MK967695	MK967696	NC_007454	NC_025355	NC_043540
MK967695 BatPV/Hipposideros/UP450/RWA/2008	76.4	63.2	75.0	75.0	71.3	83.8	73.5	73.5	74.2	72.0	72.0	73.5	86.0	72.0	83.0	79.4	87.5		73.1	66.0	68.0	67.0
MK967696 BatPV/Otomops/UP535/RWA/2008	75.7	65.4	74.2	72.7	75.0	77.2	77.2	72.0	78.6	75.0	72.0	69.8	78.6	72.7	77.9	71.3	77.9	77.9		66.3	67.5	66.3
NC_007454 J-virus	72.7	65.4	75.0	66.9	80.8	69.8	68.3	71.3	75.0	66.9	72.7	69.1	69.1	75.0	69.1	69.8	70.5	71.3	72.7		71.4	71.2
NC_025355 Tailam virus	74.2	65.4	75.7	71.3	87.5	71.3	70.5	70.5	72.0	69.1	69.8	68.3	72.7	75.0	72.7	71.3	76.4	72.7	72.7	81.6		71.7
NC_043540 Mount Mabu Lophuromys virus 2	74.2	65.4	72.7	66.9	77.2	69.1	67.6	67.6	70.5	67.6	69.1	68.3	68.3	72.0	69.1	70.5	71.3	69.1	72.0	80.1	78.6	

Sequences represented:

¹ BatPV/Hipposideros/DON009B025/BW/2021, BatPV/Hipposideros/DON009B018/BW/2021 and BatPV/Nycteris/DON010B024/BW/2021

² BatPV/Nycteris/DON007B008/BW/2020;

BatPV/Nycteris/DON007B022/BW/2020;

BatPV/Nycteris/DON007B026/BW/2020;

BatPV/Nycteris/DON007B033/BW/2020;

BatPV/Nycteris/DON007B043/BW/2020; BatPV/Nycteris/DON007B047/BW/2020

³ BatPV/Hipposideros/DON009B008/BW/2021

[†] unique sequence

BatPV/Nycteris/DON007B010/BW/2020;

BatPV/Nycteris/DON007B023/BW/2020;

BatPV/Nycteris/DON007B028/BW/2020;

BatPV/Nycteris/DON007B035/BW/2020;

BatPV/Nycteris/DON007B015/BW/2020;

BatPV/Nycteris/DON007B024/BW/2020;

BatPV/Nycteris/DON007B029/BW/2020;

BatPV/Nycteris/DON007B039/BW/2020;

BatPV/Nycteris/DON007B021/BW/2020;

BatPV/Nycteris/DON007B025/BW/2020;

BatPV/Nycteris/DON007B030/BW/2020;

BatPV/Nycteris/DON007B040/BW/2020;

3.2.1 SCR RT-PCR phylogenetic and sequence identity analyses

The sequences from the SCR assay were analyzed as in the RMH RT-PCR phylogenetic analyses. RMH sequences were also included in the multiple sequence alignment (MSA) because they share a small overlapping region with SCR assay-derived sequences. Notably, all sequences were trimmed following multiple sequence alignment to include only the overlapping smaller region within the target region amplified. The trimmed sequences were subsequently used in the construction of the phylogenetic tree.

From the phylogenetic tree, **Figure 3.3**, it was observed that the inclusion of additional sequences identified an additional clade associated with the *Nycteris* bat. Sequences derived from the *Nycteris* bat reveal consistent clustering pattern (as seen also in **Figure 3.2**) highlighting the genetic distinctiveness within the phylogenetic analysis. Within the *Nycteris* clade, the SCR RT-PCR assay derived sequences (indicated in blue) cluster together, as do those derived using the RMH RT-PCR assay, with exception of BatPV/*Nycteris*/DON007B005/BW/2020 which forms a larger clade with the sequence KC578581 BatPV/*Nycteris*/RSA-855/2010, indicating a potential relationship with *Jeilongvirus*.

Furthermore, the *Hipposideros* spp. and *Rhinolophus* spp. sequences BatPV/*Hipposideros*/DON009B005/BW/2021 and BatPV/*Rhinolophus*/DON010B023/2020, detected within this study using the SCR assay, cluster with BatPV/*Rhinolophus*/DON010B009/BW/2021 and BatPV/*Hipposideros*/DON009B008/BW/2021 sequences obtained from the RMH assay. This clustering suggests a close genetic relationship among these bat species' viral sequences. The sequences, BatPV/*Hipposideros*/DON009B008/BW/2021 and BatPV/*Rhinolophus*/DON010B009/BW/2021, which previously clustered within the putative *Jeilongvirus* clade (**Figure 3.2**) have been confirmed to indeed belong to the *Jeilongvirus* clade. Additionally, BatPV/*Rhinolophus*/DON006B030/2020, found within the *Jeilongvirus* clade, exhibits a close genetic relationship with the sequence DQ100461 Beilong virus, which is supported by the pairwise sequence identity as shown in **Table 3.8**. Consistent with the RMH phylogeny, *Nycteris* derived sequences show distant genetic relationship with sequences derived from *Hipposideros* and *Rhinolophus* bat derived sequences.

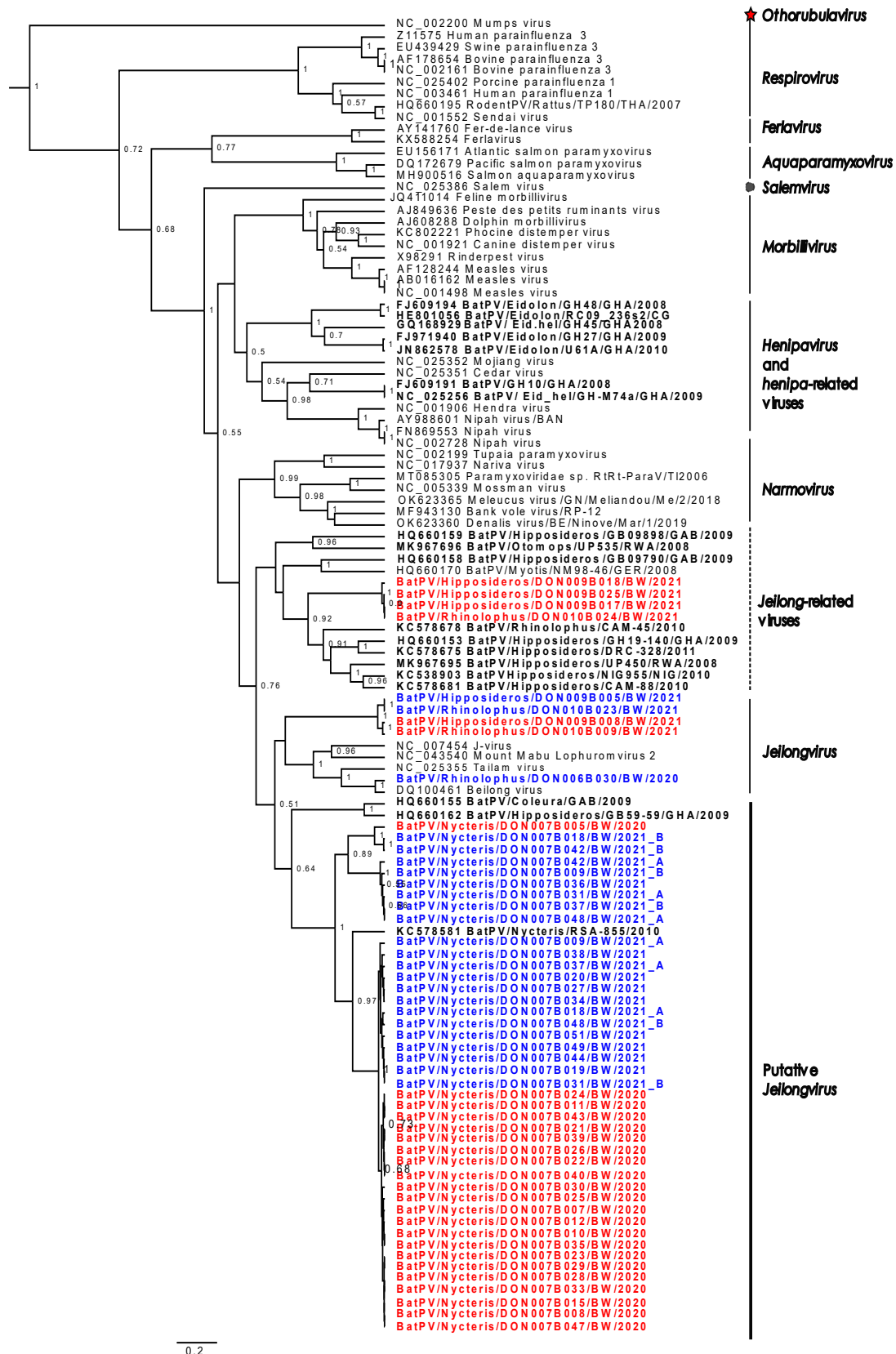


Figure 3.3: Phylogenetic analysis of Paramyxoviruses detected in bats sampled at Gcwihaba caves using the SCR assay. Bayesian phylogenetic tree constructed with 61 representative sequences obtained from GenBank identified by the ascension number, host, and location. Additionally, sequences (28) derived from the RMH assay were also included (highlighted in bold and red). Sequence length, following alignment, was trimmed to 167 nt to include maximum number of sequences for the partial polymerase (L) gene. Taxa in bold represents sequences identified in Africa, while sequences detected in this study using the SCR assay are indicated in blue and bold, with clones distinguished by the letters (A) and (B). For collapsed clades, numbers in brackets indicate the number of taxa found within the clade. Only Bayesian posterior values of ≥ 0.50 are shown at the internal nodes. The bayesian analysis was based on the TPM3uf+G substitution model. The scale bar denotes the base substitution per site. Mumps virus was selected as an outgroup for phylogeny.

Pairwise nucleotide and amino sequence identity expressed as a percentage (%) was carried out using the sequences indicated in **Table 3.5**. Due to the number of sequences detected using the SCR assay, the sequence identity is shown in two parts (**Table 3.7** and **Table 3.8**). Representative sequences were selected for inclusion for comparison with previously characterized viral sequences including those associated with bats, and those belonging to the *Jeilongvirus* genus. Full information criteria for inclusion of select detected sequences are shown in **Appendix Table 7.7**. To supplement the relationship between detected sequences with previously characterized sequences, the genetic distance is shown in **Appendix Table 7.8**.

Sequences obtained from this study and associated with *Nycteris* spp. bats, shared nucleotide identity of 75-100.0% and amino acid identity of 80-100.0%. The pairwise identity between sequences linked to *Nycteris* spp was below 70.0% for both nt and aa identity when compared to sequences associated with *Hipposideros* spp. and *Rhinolophus* spp., also obtained using the SCR assay in the current study. Most of the selected sequences obtained using the SCR assay had low nt pairwise identity scores (i.e., less than 70.0%) when compared with previously characterized sequences. The exception was observed for sequence identity compared with a previously reported sequence from South Africa (KC578581 BatPV/*Nycteris*/RSA-855/2010), with a range of 73.6-81.4% nt identity. Only two sequences (DON009B005 and DON006B030), had low aa identity when compared to the KC578581 sequence. DON009005 had the lowest nt identity score with a range of 53.2-69.4% compared to literature sequences, with the closest related sequence being HQ660159 BatPV/*Hipposideros*/GB09898/GAB/2009 at 69.4% nt identity. In comparison, DON006B30 showed highest nt identity with DQ100461 Beilong virus at 91.6% nt identity. **The similarity raises concerns about the likelihood of contamination by rodents within the cave, as Beilong virus is a rodent virus. The use of plastic sheets placed on the cave floor, though practical, may not prevent contamination by rodents. This is likely as rodents have been previously been reported to inhabit the cave (Ferguson et al., 2010).**

With regards to aa pairwise identity, the viral sequences detected in this study from *Nycteris* spp. showed a close relationship with a sequence previously reported in *Nycteris* bat in South Africa (KC578581 BatPV/*Nycteris*/RSA-855/2010). The aa identity range was between 83.6% and 92.7%. The *Rhinolophus* spp. associated sequence detected in this study (DON006B030), showed a close relationship with 87.2% aa identity with *Beilong virus* (DQ100461 Beilong virus). DON009B005, which is associated with *Hipposideros* spp. bat in this study, showed a relatedness of 70.9% aa identity with a sequence previously reported in Gabon, also associated with a *Hipposideros* bat

Table 3.7: Pairwise percent (%) sequence identity for the partial polymerase (L) gene using selected **PMV sequences (Part 1)**

Selected **PMV** sequences and sequences identified in this study (highlighted in bold and blue) were used in the calculation of pairwise identity. The nucleotide identity (upper right of the diagonal) is based on a 167-nucleotide alignment, while the amino acid identity (lower left of the diagonal) is based on a 55 amino acid alignment. For the sample derived sequences for this study, only one representative was included. Pairwise sequence identity scores of $\geq 70.0\%$ are indicated in bold for comparison between study sequences and literature sequences. For the sample derived sequences for this study, only one representative was included in the table with subsequent sequences within the cluster highlighted below the table.

Seq->	DON009B005 ¹	DON007B009_A	DON007B009_B	DON007B018_A	DON007B018_B	DON007B019 ²	DON007B022	DON007B027	DON007B031_A	DON007B036	DON007B037_A	DON007B037_B	DON007B042_A	DON007B042_B	DON007B048_A	DON007B048_B	DON007B051	DON006B030	DQ100461	HQ660153	HQ660155	HQ660158	HQ660159	HQ660162	HQ660170	HQ660195	KC538903
BatPV/Hipposideros/DON009B005/BW/2021 ¹		65.8	68.2	66.4	66.4	67.0	65.8	66.4	69.4	68.2	67.0	69.4	67.0	66.4	69.4	66.4	65.8	64.6	65.8	64.6	63.4	61.6	69.4	62.8	65.8	53.2	65.8
BatPV/Nycteris/DON007B009/BW/2021_A	60.0		80.2	98.2	76.0	98.2	98.2	98.2	79.0	79.0	98.2	79.0	77.2	76.6	79.0	96.4	97.6	64.6	67.6	67.6	67.6	65.8	65.2	67.0	60.4	52.6	66.4
BatPV/Nycteris/DON007B009/BW/2021_B	60.0	92.7		80.2	79.0	80.8	79.6	79.6	98.8	98.8	79.0	98.8	95.8	79.0	98.8	80.2	80.8	62.8	65.2	69.4	66.4	64.6	67.0	67.0	62.2	53.2	65.8
BatPV/Nycteris/DON007B018/BW/2021_A	60.0	100.0	92.7		77.2	99.4	98.8	98.2	80.2	79.0	98.8	80.2	78.4	77.8	80.2	97.6	97.6	64.6	67.6	68.2	67.6	65.8	65.2	66.4	60.4	52.0	65.8
BatPV/Nycteris/DON007B018/BW/2021_B	58.1	83.6	89.0	83.6		77.8	76.6	77.2	79.0	77.8	76.0	79.0	76.0	99.4	79.0	77.2	76.6	61.0	62.2	65.2	62.8	61.6	66.4	63.4	59.8	47.9	65.2
BatPV/Nycteris/DON007B019/BW/2021 ²	60.0	100.0	92.7	100.0	83.6		98.8	98.8	80.8	79.6	98.2	80.8	79.0	78.4	80.8	98.2	98.2	65.2	67.0	68.8	67.6	66.4	65.8	66.4	61.0	52.0	65.8
BatPV/Nycteris/DON007B022/BW/2020	60.0	100.0	92.7	100.0	83.6	100.0		97.6	79.6	78.4	97.6	79.6	77.8	77.2	79.6	97.0	97.0	64.0	67.6	68.8	68.2	65.2	65.8	67.6	61.0	53.2	67.0
BatPV/Nycteris/DON007B027/BW/2021	60.0	98.1	92.7	98.1	83.6	98.1	98.1		79.6	78.4	98.2	79.6	77.8	77.8	79.6	97.0	97.0	66.4	67.6	68.2	67.0	65.8	65.2	66.4	61.0	51.4	65.8
BatPV/Nycteris/DON007B031/BW/2021_A	60.0	90.9	98.1	90.9	87.2	90.9	90.9	90.9		98.8	79.0	100.0	97.0	79.0	100.0	80.2	79.6	64.0	65.8	70.6	66.4	65.2	67.0	67.0	62.8	53.2	65.8
BatPV/Nycteris/DON007B036/BW/2021_	58.1	89.0	96.3	89.0	85.4	89.0	89.0	89.0	98.1		77.8	98.8	95.8	77.8	98.8	79.0	79.6	62.8	65.2	69.4	65.8	64.6	66.4	66.4	62.2	53.2	65.2
BatPV/Nycteris/DON007B037/BW/2021_A	60.0	98.1	90.9	98.1	81.8	98.1	98.1	96.3	90.9	89.0		79.0	77.2	76.6	79.0	96.4	96.4	64.6	67.6	67.6	67.6	65.2	65.2	66.4	59.8	50.8	65.8
BatPV/Nycteris/DON007B037/BW/2021_B	60.0	90.9	98.1	90.9	87.2	90.9	90.9	90.9	100.0	98.1	90.9		97.0	79.0	100.0	80.2	79.6	64.0	65.8	70.6	66.4	65.2	67.0	67.0	62.8	53.2	65.8
BatPV/Nycteris/DON007B042/BW/2021_A	56.3	85.4	90.9	85.4	81.8	85.4	85.4	85.4	92.7	90.9	85.4	92.7		76.0	97.0	77.8	77.8	62.2	64.0	68.2	64.6	63.4	65.2	65.2	61.0	53.2	64.0
BatPV/Nycteris/DON007B042/BW/2021_B	58.1	83.6	89.0	83.6	100.0	83.6	83.6	83.6	87.2	85.4	81.8	87.2	81.8		79.0	77.8	77.2	61.6	62.8	65.8	63.4	62.2	66.4	63.4	60.4	47.9	65.2
BatPV/Nycteris/DON007B048/BW/2021_A	60.0	90.9	98.1	90.9	87.2	90.9	90.9	90.9	100.0	98.1	90.9	100.0	92.7	87.2		80.2	79.6	64.0	65.8	70.6	66.4	65.2	67.0	67.0	62.8	53.2	65.8
BatPV/Nycteris/DON007B048/BW/2021_B	60.0	96.3	89.0	96.3	80.0	96.3	96.3	94.5	87.2	85.4	94.5	87.2	81.8	80.0	87.2		96.4	64.0	65.8	68.8	67.6	64.6	64.6	65.8	59.8	50.8	65.8
BatPV/Nycteris/DON007B051/BW/2021	60.0	100.0	92.7	100.0	83.6	100.0	100.0	98.1	90.9	89.0	98.1	90.9	85.4	83.6	90.9	96.3		64.0	66.4	67.0	66.4	65.8	64.6	65.2	60.4	51.4	65.2
BatPV/Rhinolophus/DON006B030/2020	58.1	61.8	60.0	61.8	61.8	61.8	61.8	61.8	60.0	58.1	61.8	60.0	58.1	61.8	60.0	58.1	61.8		91.6	58.6	57.4	58.0	56.2	56.8	58.6	47.9	55.6
DQ100461 Beilong virus	61.8	67.2	65.4	67.2	67.2	67.2	67.2	67.2	65.4	63.6	67.2	65.4	63.6	67.2	65.4	63.6	67.2	87.2		62.2	61.0	60.4	58.6	61.0	61.6	52.0	59.8
HQ660153 BatPV/Hipposideros/GH19-140/GHA/2009	60.0	63.6	61.8	63.6	56.3	63.6	63.6	63.6	61.8	60.0	63.6	61.8	58.1	56.3	61.8	63.6	63.6	61.8	65.4		69.4	68.2	69.4	69.4	64.0	49.1	76.6
HQ660155 BatPV/Coleura/GAB/2009	61.8	69.0	69.0	69.0	61.8	69.0	69.0	69.0	69.0	67.2	69.0	69.0	63.6	61.8	69.0	69.0	69.0	60.0	63.6	72.7		62.2	65.8	85.0	59.8	47.3	71.2
HQ660158 BatPV/Hipposideros/GB09790/GAB/2009	60.0	61.8	61.8	61.8	54.5	61.8	61.8	61.8	63.6	61.8	61.8	63.6	60.0	54.5	63.6	58.1	61.8	58.1	61.8	70.9	61.8		65.8	62.2	69.4	48.5	68.2
HQ660159 BatPV/ Hipposideros/GB09898/GAB/2009	70.9	70.9	69.0	70.9	65.4	70.9	70.9	69.0	69.0	67.2	72.7	69.0	63.6	65.4	69.0	67.2	70.9	61.8	65.4	65.4	65.4	67.2		66.4	61.6	50.8	71.8
HQ660162 BatPV/Hipposideros/GB59-59/GHA/2009	58.1	65.4	65.4	65.4	61.8	65.4	65.4	65.4	65.4	63.6	65.4	65.4	61.8	61.8	65.4	65.4	65.4	58.1	61.8	72.7	92.7	61.8	63.6		58.6	50.8	71.8

Seq->	DON009B005 ¹	DON007B009_A	DON007B009_B	DON007B018_A	DON007B018_B	DON007B019 ²	DON007B022	DON007B027	DON007B031_A	DON007B036	DON007B037_A	DON007B037_B	DON007B042_A	DON007B042_B	DON007B048_A	DON007B048_B	DON007B051	DON006B030	DQ100461	HQ660153	HQ660155	HQ660158	HQ660159	HQ660162	HQ660170	HQ660195	KC538903
HQ660170 BatPV/ Myotis/NM98-46/GER/2008	61.8	60.0	61.8	60.0	61.8	60.0	60.0	60.0	63.6	61.8	60.0	63.6	60.0	61.8	63.6	56.3	60.0	54.5	58.1	63.6	56.3	76.3	65.4	58.1		50.8	63.4
HQ660195 RodentPV/Rattus/TP180/THA/2007	34.5	38.1	36.3	38.1	36.3	38.1	38.1	38.1	38.1	36.3	38.1	38.1	38.1	36.3	38.1	34.5	38.1	36.3	41.8	32.7	34.5	36.3	38.1	34.5	38.1		50.8
KC538903 BatPV/NIG955/NIG/2010	67.2	65.4	63.6	65.4	58.1	65.4	65.4	65.4	63.6	61.8	65.4	63.6	60.0	58.1	63.6	65.4	65.4	61.8	65.4	83.6	72.7	70.9	67.2	72.7	65.4	32.7	

Sequences within the same cluster:

¹BatPV/Rhinolophus/DON010B023/BW/2021

²BatPV/Nycteris/DON007B020/BW/2021, BatPV/Nycteris/DON007B031/BW/2021_B, BatPV/Nycteris/DON007B034/BW/2021, BatPV/Nycteris/DON007B038/BW/2021, BatPV/Nycteris/DON007B044/BW/2021 and BatPV/Nycteris/DON007B049/BW/2021

Table 3.8: Pairwise percent (%) sequence identity for the partial polymerase (L) gene using selected PMV sequences (Part 2)

(Continuation of sequence identity determination using the remaining select PMV sequences).

Seq->	DON009B005 ¹	DON007B009_A	DON007B009_B	DON007B018_A	DON007B018_B	DON007B019 ²	DON007B022	DON007B027	DON007B031_A	DON007B036	DON007B037_A	DON007B037_B	DON007B042_A	DON007B042_B	DON007B048_A	DON007B048_B	DON007B051	DON006B030	KC578581	KC578675	KC578678	KC578681	MK967695	MK967696	NC_007454	NC_025355	NC_043540
BatPV/Hipposideros/DON009B005/BW/2021 ¹		65.8	68.2	66.4	66.4	67.0	65.8	66.4	69.4	68.2	67.0	69.4	67.0	66.4	69.4	66.4	65.8	64.6	63.4	64.0	68.8	65.8	64.6	61.6	66.4	65.8	61.0
BatPV/Nycteris/DON007B009/BW/2021_A	60.0		80.2	98.2	76.0	98.2	98.2	98.2	79.0	79.0	98.2	79.0	77.2	76.6	79.0	96.4	97.6	64.6	81.4	68.2	66.4	70.6	70.0	64.6	66.4	69.4	64.0
BatPV/Nycteris/DON007B009/BW/2021_B	60.0	92.7		80.2	79.0	80.8	79.6	79.6	98.8	98.8	79.0	98.8	95.8	79.0	98.8	80.2	80.8	62.8	77.2	68.8	68.2	69.4	68.2	64.6	64.0	67.0	59.8
BatPV/Nycteris/DON007B018/BW/2021_A	60.0	100.0	92.7		77.2	99.4	98.8	98.2	80.2	79.0	98.8	80.2	78.4	77.8	80.2	97.6	97.6	64.6	80.2	67.0	65.8	70.0	70.0	65.2	65.8	68.8	62.8
BatPV/Nycteris/DON007B018/BW/2021_B	58.1	83.6	89.0	83.6		77.8	76.6	77.2	79.0	77.8	76.0	79.0	76.0	99.4	79.0	77.2	76.6	61.0	73.6	62.8	61.0	67.0	66.4	66.4	63.4	69.4	61.0
BatPV/Nycteris/DON007B019/BW/2021 ²	60.0	100.0	92.7	100.0	83.6		98.8	98.8	80.8	79.6	98.2	80.8	79.0	78.4	80.8	98.2	98.2	65.2	80.2	67.0	65.8	70.0	70.6	65.2	65.2	69.4	62.8
BatPV/Nycteris/DON007B022/BW/2020	60.0	100.0	92.7	100.0	83.6	100.0		97.6	79.6	78.4	97.6	79.6	77.8	77.2	79.6	97.0	97.0	64.0	81.4	68.2	67.0	71.2	70.6	66.4	65.8	68.2	62.8
BatPV/Nycteris/DON007B027/BW/2021	60.0	98.1	92.7	98.1	83.6	98.1	98.1		79.6	78.4	98.2	79.6	77.8	77.8	79.6	97.0	97.0	66.4	80.8	67.0	67.0	70.0	70.0	64.6	65.2	70.0	62.8
BatPV/Nycteris/DON007B031/BW/2021_A	60.0	90.9	98.1	90.9	87.2	90.9	90.9	90.9		98.8	79.0	100.0	97.0	79.0	100.0	80.2	79.6	64.0	77.8	69.4	69.4	69.4	68.8	65.2	64.0	67.6	60.4
BatPV/Nycteris/DON007B036/BW/2021_	58.1	89.0	96.3	89.0	85.4	89.0	89.0	89.0	98.1		77.8	98.8	95.8	77.8	98.8	79.0	79.6	62.8	77.2	68.8	68.2	68.8	68.2	64.0	64.0	67.0	59.8
BatPV/Nycteris/DON007B037/BW/2021_A	60.0	98.1	90.9	98.1	81.8	98.1	98.1	96.3	90.9	89.0		79.0	77.2	76.6	79.0	96.4	96.4	64.6	80.8	67.0	65.8	70.0	69.4	64.6	65.8	68.8	62.8
BatPV/Nycteris/DON007B037/BW/2021_B	60.0	90.9	98.1	90.9	87.2	90.9	90.9	90.9	100.0	98.1	90.9		97.0	79.0	100.0	80.2	79.6	64.0	77.8	69.4	69.4	69.4	68.8	65.2	64.0	67.6	60.4
BatPV/Nycteris/DON007B042/BW/2021_A	56.3	85.4	90.9	85.4	81.8	85.4	85.4	85.4	92.7	90.9	85.4	92.7		76.0	97.0	77.8	77.8	62.2	74.8	67.0	68.2	67.6	67.0	63.4	63.4	65.8	59.8
BatPV/Nycteris/DON007B042/BW/2021_B	58.1	83.6	89.0	83.6	100.0	83.6	83.6	83.6	87.2	85.4	81.8	87.2	81.8		79.0	77.8	77.2	61.6	74.2	62.8	61.0	67.0	67.0	66.4	63.4	69.4	61.6

Seq->	DON009B005 ¹	DON007B009_A	DON007B009_B	DON007B018_A	DON007B018_B	DON007B019 ²	DON007B022	DON007B027	DON007B031_A	DON007B036	DON007B037_A	DON007B037_B	DON007B042_A	DON007B042_B	DON007B048_A	DON007B048_B	DON007B051	DON006B030	KC578581	KC578675	KC578678	KC578681	MK967695	MK967696	NC_007454	NC_025355	NC_043540
BatPV/Nycteris/DON007B048/BW/2021_A	60.0	90.9	98.1	90.9	87.2	90.9	90.9	90.9	100.0	98.1	90.9	100.0	92.7	87.2		80.2	79.6	64.0	77.8	69.4	69.4	69.4	68.8	65.2	64.0	67.6	60.4
BatPV/Nycteris/DON007B048/BW/2021_B	60.0	96.3	89.0	96.3	80.0	96.3	96.3	94.5	87.2	85.4	94.5	87.2	81.8	80.0	87.2		96.4	64.0	79.6	67.0	65.8	70.0	70.6	64.0	64.0	68.8	62.2
BatPV/Nycteris/DON007B051/BW/2021	60.0	100.0	92.7	100.0	83.6	100.0	100.0	98.1	90.9	89.0	98.1	90.9	85.4	83.6	90.9	96.3		64.0	79.0	65.8	64.6	69.4	70.6	63.4	65.2	68.8	61.6
BatPV/Rhinolophus/DON006B030/2020	58.1	61.8	60.0	61.8	61.8	61.8	61.8	61.8	60.0	58.1	61.8	60.0	58.1	61.8	60.0	58.1	61.8		61.6	60.4	58.6	58.0	59.8	62.2	65.8	72.4	59.8
KC578581 BatPV/Nycteris/RSA-855/2010	58.1	92.7	90.9	92.7	85.4	92.7	92.7	92.7	90.9	89.0	92.7	90.9	83.6	85.4	90.9	89.0	92.7	61.8		66.4	65.2	71.2	69.4	63.4	62.2	67.6	63.4
KC578675 BatPV/Hipposideros/DRC-328/2011	60.0	61.8	60.0	61.8	54.5	61.8	61.8	61.8	60.0	58.1	61.8	60.0	56.3	54.5	60.0	61.8	61.8	63.6	58.1		73.0	77.2	73.6	68.8	62.8	64.0	64.0
KC578678 BatPV/Rhinolophus/CAM-45/2010	63.6	63.6	61.8	63.6	52.7	63.6	63.6	65.4	61.8	60.0	63.6	61.8	60.0	52.7	61.8	63.6	63.6	56.3	60.0	72.7		75.4	71.8	62.2	59.8	60.4	65.2
KC578681 BatPV/Hipposideros/CAM-88/2010	65.4	69.0	67.2	69.0	58.1	69.0	69.0	69.0	67.2	65.4	69.0	67.2	63.6	58.1	67.2	69.0	69.0	60.0	65.4	80.0	78.1		81.4	71.8	63.4	65.8	62.8
MK967695 BatPV/Hipposideros/UP450/RWA/2008	63.6	70.9	67.2	70.9	58.1	70.9	70.9	69.0	69.0	67.2	70.9	69.0	65.4	58.1	69.0	70.9	70.9	58.1	65.4	80.0	78.1	89.0		66.4	63.4	65.8	62.2
MK967696 BatPV/Otomops/UP535/RWA/2008	60.0	67.2	65.4	67.2	61.8	67.2	67.2	67.2	65.4	63.6	67.2	65.4	61.8	61.8	65.4	63.6	67.2	63.6	65.4	70.9	60.0	74.5	69.0		61.6	62.2	59.2
NC_007454 J-virus	56.3	70.9	67.2	70.9	63.6	70.9	70.9	69.0	69.0	67.2	70.9	69.0	67.2	63.6	69.0	67.2	70.9	67.2	69.0	56.3	56.3	60.0	61.8	60.0		70.6	70.0
NC_025355 Tailam virus	61.8	69.0	67.2	69.0	63.6	69.0	69.0	69.0	69.0	67.2	69.0	69.0	67.2	63.6	69.0	65.4	69.0	76.3	70.9	60.0	61.8	63.6	65.4	63.6	78.1		66.4
NC_043540 Mount Mabu Lophuromys virus 2	56.3	61.8	60.0	61.8	61.8	61.8	61.8	61.8	61.8	60.0	61.8	61.8	61.8	61.8	61.8	58.1	61.8	63.6	61.8	54.5	61.8	54.5	54.5	60.0	74.5	70.9	

Sequences within the same cluster:

¹ BatPV/Rhinolophus/DON010B023/BW/2021

² BatPV/Nycteris/DON007B020/BW/2021, BatPV/Nycteris/DON007B031/BW/2021_B, BatPV/Nycteris/DON007B034/BW/2021, BatPV/Nycteris/DON007B038/BW/2021, BatPV/Nycteris/DON007B044/BW/2021 and BatPV/Nycteris/DON007B049/BW/2021

Chapter 4: Discussion

Studies on the detection and prevalence of viral pathogens such as coronaviruses and paramyxoviruses using conventional PCR in Sub-Saharan Africa have yielded preliminary results that have been the foundation of surveillance projects in some countries. In southern Africa, coronaviruses (CoV) and paramyxoviruses (PMV) have been identified in bats in South Africa (Corman et al., 2014; Geldenhuys et al., 2018; Mortlock et al., 2015), Mozambique (Hoarau et al., 2021; Léa Joffrin et al., 2020; Vanmechelen et al., 2018), Zambia (Muleya et al., 2014), and Zimbabwe (Chidoti et al., 2022). Given the geographical proximity of these countries to Botswana, and therefore possible migratory behaviour of bats across borders, it is highly likely that bats in Botswana harbour the same or similar viral pathogens. The current study was therefore conducted, as the first in Botswana, to determine the presence of CoV and PMV within bats in Botswana.

4.1 Presence of CoV and PMV viral RNA in bat fecal samples

Fecal samples from bats, a non-invasive sampling technique, have previously been used in various studies for the detection of viral nucleic acids (Bourgarel et al., 2018; Chidoti et al., 2022; Hardmeier et al., 2021; Li et al., 2010; Pauly et al., 2017; Zheng et al., 2018). In fact, approximately 70 of studies on biosurveillance of viruses in bats, specifically relating to coronaviruses, used bats fecal matter, compared to invasive techniques such as gastrointestinal tissues or rectal and mouth swabs (Geldenhuys et al., 2021). The current study used a similar approach to determine the presence of coronaviruses and paramyxoviruses within fecal samples collected from the Gcwihaba caves, in the Northwestern region of Botswana. Of the 173 samples analyzed, 2.31% ($n = 4/173$) were positive for CoV RNA, whilst 30.41% ($n = 52$) were positive for PMV. In a study conducted in Zimbabwe, where similar fecal samples were collected from similar bat species, during the same period of March was found to be 34.75% (Chidoti et al., 2022), which was significantly higher than in the present study. The study collected fecal samples using 2m² plastic sheet placed on the cave floor with an individual bat sample pellet collected with a 20cm-by-20cm area. Other studies including insectivorous bats showed similar trends using similar sampling methods (Berto et al., 2018; Rizzo et al., 2017; Valitutto et al., 2020). On the other hand, PMV prevalence from fecal samples in various studies is dependent on the type of sample being analyzed. For example, a study by Berto et al., (2018) observed PMV RNA prevalence of 1.6% in pooled feces and 4.9% for pooled urine. Contrary to the low detection levels reported in this

study and the aforementioned, a study by Baker et al., (2012) demonstrated a higher positivity of 43% for pooled urine, although that could have been an overestimation if the values were not corrected for pooling. However, the differences in sampling methodologies, notably the individual sampling approach from the various studies versus the pooled sampling approach in the present study, might explain the variation in detection rates. Individual sampling strategies, such as those employed in the Zimbabwe study, likely reduced the likelihood of urine-fecal cross-contamination and ensured that the fecal samples were more representative of individual bats. On the other hand, the current study's sampling strategy and the possibility of urine-fecal contamination could have led to a dilution of the fecal samples' detection rate, which would have decreased the observed prevalence of CoV viral RNA. In the case of PMV detection, sampling methodology may similarly contribute to lower detection rate. Additionally, urine has been noted as the predominantly used and suitable sample for viral RNA detection (Rizzo et al., 2017)

Seasonal variation in viral RNA prevalence is a key element that has recently been incorporated in surveillance studies. Preliminary observations of variation in viral prevalence relative to season was determined by collecting samples in the dry-cold and wet-hot seasons for this study. There was a notable difference in the positivity of the samples during the different seasons observed in the detection rate of PMV and CoV in bats, where CoV positivity was noted in the wet season, whereas PMV detection was observed for both seasons, but the proportion for the latter being higher in the dry season. It is important to note that the findings on detection rate may have been influenced by the seasonal preference of the bat species within the cave. The dominant species during the wet season were suspected to be *Rhinolophus* spp. and *Hipposideros* spp. while the dry season samples were predominantly comprised of *Rhinolophus* spp. and *Nycteris* spp. In a study covering bats of Mozambique, which include members of the bat families Hipposideridae, Miniopteridae, Molossidae, Nycteridae, Rhinolophidae and Rhinonycteridae, CoV prevalence was reportedly higher during the wet season (Joffrin et al., 2022), and similarly for a study carried out in Gabon in Hipposideridae and Miniopteridae bats (Maganga et al., 2020). With regards to PMV seasonal prevalence, Mélade et al., (2016) outlines the impact of temperature and rainfall on PMV viral transmission, with higher temperatures and high average rainfall facilitating high transmission and subsequently detection. Furthermore, PMV shedding appears to be heightened during the dry season as noted by Mortlock et al., (2021), while CoV shedding is higher during the wet season in Africa as reported by Lane et al., (2022). However, the studies specifically investigated different bat species (PV detection in *R. aegyptiacus*, and CoV detection in Hipposideridae Pteropodidae, Molossidae), thus these observations may not

directly apply to all bat species, including the suspected bat species inhabiting the Gcwihaba cave. Different species may exhibit different shedding patterns influenced by various factors including bat reproductive stages and seasonal variations. This is clearly evident from the study carried out by Chidoti et al., (2022), where prevalence according to species and reproductive stage was determined to be high in *Macronycteris gigas* during the juvenile, lactation and weaning stage, compared to *Hipposideros* spp., *Nycteris* spp. *Rhinolophus* spp. and *Miniopterus* spp. during gestation, parturition and juvenile stage. As viral shedding may have seasonal variations, the preliminary findings of the study indicate the necessity of carrying out spatiotemporal studies to understand the shedding patterns of coronavirus and paramyxovirus circulating in the Botswana bats.

In addition to sample type and season, there are other factors that play a role in predicting viral prevalence in bats. These include resource availability, bat species, age, and sex (Edson et al., 2015; Giles et al., 2018; Lane et al., 2022; McFarlane et al., 2011; Plowright et al., 2016; Ruiz-Aravena et al., 2022). The Gcwihaba caves are known to harbor several bat species, including those belonging to the families *Rhinolophus* spp. *Hipposideros* spp. and *Nycteris* spp. However, the identity of these bats was presumptive and based on gross morphological observations. Based on this presumptive identity, CoV RNA was detected in *Rhinolophus* spp., whilst PMV RNA was detected in all the three bat species found in the cave. Due to the pooling of the fecal samples during collection, where the under-roost sampling technique was employed, it cannot be ascertained conclusively if the proposed bat species alone contributed to the positive viral RNA detected. This identity uncertainty can be resolved through genetic identification of bats to species level, using mitochondrial gene segments that include the cytochrome b (*cytB*) and cytochrome c oxidase subunit I (COI), which have been used extensively for the identification of bats (Chidoti et al., 2022; George et al., 2022; Markotter et al., 2019; Sasaki et al., 2012; Valitutto et al., 2020). However, it is crucial to emphasize that genetic identification from mixed fecal samples, as gathered in this study, might be difficult due to possible contamination and the inclusion of genetic material from multiple bats. This would require adjustment of the sample methodology where individual pellets are collected instead of a homogeneous sample to ensure minimum contamination. Species identification of bats was conducted based on morphological characteristics observed in the field, as detailed identification techniques were beyond the scope of this study.

The under-roost sampling technique employed in this study has previously been used in similar studies where CoV and PMV were investigated in urine and fecal samples from bats (Berto et al., 2018; Bing Chua, 2003; Chua, 2003; Field et al., 2015). One limitation with this method is the placement and duration between plastic sheets setup and time of fecal

collection. Fofanov et al., (2018) has outlined that there are problems associated with microbiota diversity of bat feces, particularly the time between host defecation and sample stabilization (sample preservation to avoid degradation). Prolonged exposure between these times exposes the samples to environmental conditions. Biases may be introduced in the detection of viruses, particularly in the case of PMV. PMV detection is higher in urine samples of insectivorous bats as observed in studies by Berto et al., (2018), Rizzo et al., (2017) and Muzeniek et al., (2022). Additionally, studies by Dietrich et al., (2017) and Dietrich & Markotter (2019) report increased niche specialization in urine compared to fecal samples by microbiota. This suggests that prolonged sampling times may increase the risk of urine-fecal contamination, as urine droplets were observed on some of the plastic sheets on which fecal pellets had collected in the present investigation. However, it is important to note that the studies by Dietrich et al. and similar studies are biased towards impact of sample type and sampling approach on bacterial diversity. Therefore, a refinement of the sampling method for future studies in Botswana would be necessary. Studies by Baker et al., (2012) indicate one hour interval between sheet placement and sample collection as sufficient, or alternatively, placement just before dusk and sampling done at sunrise, (Bourgarel et al., 2018; Joffrin et al., 2022; Peel et al., 2019). Nevertheless, as highlighted by Giles et al., (2021), pooled under-roost sampling, offers an advantage over individual sampling (capture and release), particularly where viral prevalence is unknown. This was particularly relevant to the current investigation which is the first of its kind in Botswana, where knowledge of viral and bat diversity is limited. Further studies with modified methods as suggested by Giles et al., (2021), such as reduction in sheet size, number and distance placed, and dispersion distance between sheets, would need to be considered.

There are various factors to consider when interpreting the findings of this study on the proportion of positive viral RNA samples. In particular, the limitations of sample pooling, urine contamination, and co-roosting can significantly impact the identification of paramyxovirus and coronavirus in bat fecal samples obtained from the Gcwihaba caves. When samples are pooled, the viral RNA can become diluted, leading to decreased detection sensitivity (Grobe et al., 2021; Jayakody et al., 2022). Additionally, urine may reveal a higher detection rate compared to fecal samples, depending on virus species or family, which can impact the likelihood of false-positives. Finally, co-roosting among multiple bat species can complicate the identification of viral sources since viruses may originate from various hosts. It is therefore crucial to implement accurate sampling techniques to accurately assess viral prevalence and diversity in bat populations, taking these limitations into account.

4.2 CoV and PMV phylogeny and identity analyses

This study focused on utilizing conserved genes, specifically, the RNA-dependent RNA polymerase (RdRp) for coronaviruses and large protein (L) for paramyxoviruses to conduct phylogenetic analyses. These conserved genes serve as molecular markers for conducting detailed phylogenetic analysis. Through the phylogenetic analysis, the taxonomic positioning and genetic relationship of the viral sequences detected from the fecal samples was determined. This entailed comparative analysis of the phylogenetic relationships derived from the viral RNA positive samples with previously published sequences available on the NCBI database. Such a comparison facilitates a comprehensive understanding of how the detected viral sequences relate to those previously characterized, and subsequently provides insight into the evolutionary dynamics and potential implications for zoonotic transmission within Botswana, and possibly its neighbouring countries.

The phylogenetic identification of CoV viral RNA detected in fecal samples collected from bats inhabiting the Gcwihaba caves shows clustering in the *Betacoronavirus* subfamily, specifically the subgenus *Merbecovirus* (**Figure 3.1**). Sequences from the Gcwihaba caves cluster with sequences previously identified in bats found in Zimbabwe (in *Nycteris*, *Hipposideros* and *Rhinolophus* bats) and South Africa (in *Miniopterus* bats) suggesting a high degree of relatedness. Notably, the bat host from which the CoV RNA was detected was horseshoe bats (*Rhinolophus* spp.). This is not the first instance where merbecoviruses has been associated from a *Rhinolophus* bat. The virus has previously been reported in fecal samples of *Rhinolophus* sp. in Zimbabwe (Chidoti et al., 2022). Interestingly, most of the detected coronaviruses in the *Rhinolophus* spp., are SARS-related coronaviruses (*Sarbecovirus*), such as the virus strains detected in rectal swabs of Kenyan *Rhinolophus* sp. bats (Tao & Tong, 2019) and Rwandan *Rhinolophus clivosus* bats (Markotter et al., 2019). Other reports of SARS-related coronaviruses in *Rhinolophus* bats have been made in studies in Southeast Asia including Thailand (Wacharapluesadee et al., 2021), Japan in *Rhinolophus cornutus* bats (Murakami et al., 2020) and China (Ge et al., 2013; Hu et al., 2017; Temmam et al., 2022). Nonetheless, the presence of merbecoviruses instead of sarbecoviruses in the Gcwihaba *Rhinolophus* bats may be due to the former's use of general receptors such as dipeptidyl peptidase-4 (DPP4) (Cui et al., 2013; Lau, Fan, et al., 2018; Lau, Zhang, et al., 2018; Luo et al., 2018) which is a conserved receptor and available in a wider host range. Raj et al., (2013) suggests that the availability and conserved nature of DPP4 within the mammalian host has the potential to facilitate host switching which could explain the detection of merbecoviruses within the *Rhinolophus* bats as observed in this study. Few merbecoviruses are identified generally in bat surveillance studies thus limited information is available to support this

hypothesis. The wide host range in the *Merbecovirus* clade as shown by the CoV phylogeny, and further findings by various studies including Annan et al., (2013), Anthony et al., (2017), and Hashemi-Shahraki et al., (2013), emphasize the potential for viral sharing among different bat species. Additionally, the CoV assay derived sequences also reveal some degree of genetic relatedness to bat associated MERS-related viruses reported in South Africa, Uganda and China (as shown in **Table 3.3**). This is to be expected as merbecoviruses are grouped with MERS-related viruses within the *Betacoronavirus* subgroup (Corman et al., 2014; Lau et al., 2013; Tolentino et al., 2024).

Though the merbecoviruses were associated with *Rhinolophus* spp. in the current study, it is important to take into consideration that cave environments may host multiple bat species (Barros et al., 2021), as is the case with the Gcwihaba caves. A previous study by Annan et al., (2013) identified the high prevalence of similar betacoronaviruses within *Nycteris cf. gambiensis*, and due to the sampling technique used, the likelihood of contribution by the *Nycteris* sp. within the cave cannot be overlooked. It is also crucial to acknowledge the possibility of contributions from other bat species in the area, especially potentially through fly-bys. Furthermore, the single detection of a virus does not always suggest active replication within the bat host (Markotter et al., 2020). For instance, the reported sequence from *Rhinolophus* bat in Zimbabwe (Chidoti et al., 2022) may have come from environmental samples rather than a specific individual, as such confirmation requires individual bat sampling as was done in a study in Ghana (Suu-Ire et al., 2022). This is also the case for this study as under-roost pooled samples were used instead of individual bat sampling. Consequently, to strengthen the analysis, it is essential to consider confirmatory detections, as a single detection may result from contact with an infected individual instead of active replication. Confirmatory studies may include viral load quantification, as was demonstrated with *Nycteris* samples in Ghana (Annan et al., 2013). Other measures to study the patterns of viral load is through longitudinal sampling (Chidoti et al., 2022; Geldenhuys et al., 2023; Wacharapluesadee et al., 2018). Additionally, incorporating species-specific confirmatory methods would ensure that the viral genetic material is accurately attributed to the relevant host species.

In the case of the **PMV** RNA detection and identification, the RMH assay phylogeny clustering indicates that *Rhinolophus* spp. and *Hipposideros* spp. associated viral RNA sequences cluster within the Jeilongvirus-related clade with previously reported sequences from Cameroon, Ghana, DRC and Rwanda (as shown in **Figure 3.2**). Furthermore, the *Nycteris* spp. associated sequences clustered with putative *Jeilongvirus* viruses (**Figure 3.2**). There are previous reports associating jeilongviruses with the *Hipposideros* bat in China (Wu et al.,

2016; Zhu et al., 2022) and Malaysia (Wells et al., 2022). The three bat genera highlighted within the study (*Nycteris*, *Hipposideros* and *Rhinolophus*) are insectivorous bats, and have been found to have viral **PMV** RNA in southern, eastern, and central Africa. A study by Mortlock et al., (2015), demonstrated that viruses within the subgenera *Morbillivirus*, *Jeilongvirus* and related viruses were likely to be detected within insectivorous bats, consistent with present findings. Nucleotide sequence identity comparison of **PMV** sequences detected using the RMH with known and classified paramyxoviruses was observed as indicated in **Table 3.6**, with the highest score (88.2% aa; 76.5% nt) being that of *KC578581 BatPV/Nycteris/RSA-855/2010* of which *Nycteris thebaica* is the host. The host species of the **PMV** sequences that shared this identity were also of the *Nycteris* sp. suggesting circulation of related virus within this species in the region.

The SCR assay, a relatively new assay developed by Vanmechelen et al., (2022), was used for samples that were inconclusive when the RMH assay was used. The SCR assay has the added advantage of **increasing the number and diversity of viruses that can be detected in the subfamily Orthoparamyxovirinae. It is important to note that the Jeilong viral genus was not known at the time when the RMH test was developed. Therefore, while both assays have their strengths and limitations, the SCR assay offers an expanded scope of detection within the Orthoparamyxovirinae subfamily compared to the RMH assay.** Though the SCR encompasses detection of viruses within the subfamily, this is at the cost of the final nested product size due to the primer design, however the two assays are complimentary as they target overlapping regions of the L protein. Furthermore, Vanmechelen et al., (2022) employed the two assays for the detection of paramyxoviruses and demonstrated that the SCR assay had a lower false negative rate when compared to the RMH assay. In the partial L gene phylogenetic analyses for the SCR assay-derived sequences, only one sample sequence (BatPV/Rhinolophus/DON006B030) clustered within the *Jeilongvirus* clade, as shown in **Figure 3.3**. This sequence was found to be closely related to Beilong virus (*Beilong jeilongvirus*), with 91.6% nt and 87.2% aa identity. Beilong virus was initially associated with rodents (Vanmechelen et al., 2018; Wilkinson et al., 2014; Woo et al., 2012; Wu et al., 2018) and shrews (Chen et al., 2020; Onyuok et al., 2019). However, bat-associated jeilongviruses have also been reported in studies by Bourgarel et al., (2018), Mortlock et al., (2019), Markotter et al., (2019) and Waruhiu et al., (2017).

The presence of a rodent-associated viral pathogen in insectivorous bats suggests interaction (direct or indirect) between the two mammalian species, although few reports verify this hypothesis. **However, this discovery should be regarded cautiously, given the risk of rodent contamination in the cave environment. The high genetic relatedness based on the sequence**

identity to the sequence to Beilong virus, a single positive sample similar to Beilong virus, suggests a likelihood of rodent contamination. Porcupine quills were spotted on the cave floor during sampling, which indicates the presence of rodents within Gcwihaba cave, further supporting the possibility of contamination. The sampling methods, which included the use of plastic sheets laid on the cave floor, further increases the likelihood of contamination from rodents. Some bat species, such as *Desmodus rotundus* and *Pteronotus alitus*, have been found to harbor viruses closely related to Beilong viruses (Darcissac et al., 2021). In addition, the findings of the SCR phylogeny suggest that a single putative species may have been found using the SCR assay. The viral sequences detected in this study from *Rhinolophus* bat, BatPV/Rhinolophus/DON006B030/BW/2020, showed high relatedness phylogenetically and identity wise to Beilong virus within the *Jeilongvirus* genus is likely to be a putative jeilong species than the viral sequences detected associated with the *Nycteris* bat. However, to conclusively infer that the sequence is a putative species, further genetic characterization is required. A putative species similar to Beilong virus has been previously described in *Hipposideros ruber* in South Africa (Markotter et al., 2019).

The analysis of the RMH and SCR phylogenetic trees revealed a close association between sequences of viruses in *Rhinolophus* spp. and *Hipposideros* spp. bats, which suggest possible viral sharing within the two species within the cave and possibly those within the Sub-Saharan region. A study by Chidoti et al., (2022) described similar findings of viral sharing between the same bat species, which supports the hypothesis of virus sharing within the cave, though also highlighting the likelihood of cross-contamination linked to the sampling method. Willoughby et al., (2017) elaborated on the likelihood of viral sharing among cave-dwelling bats particularly in phylogenetically related bat species. Chornelia & Hughes (2022) emphasized the relatedness of Rhinolophidae and Hipposideridae bat families, additionally pointing out their similarity in geographic distribution. This shared geography could explain the cohabitation of both species in the same roosting habitat and the possible viral sharing that may arise as a result. Additionally, Mortlock et al., (2015) underscored the co-evolutionary relationship between paramyxoviruses and their bat hosts, revealing the connection between viral incidence within bat species and the differentiation between natural (true) hosts and incidental hosts. In this context, it is likely that *Nycteris* bat is a true host for the putative *Jeilongvirus*, while *Rhinolophus* spp. and *Hipposideros* spp. are incidental hosts for Jeilong-related viruses.

Interestingly, **Figure 3.3** shows a variation to those in **Figure 3.2** in the **PMV** sequences detected by the SCR and RMH assays for sequences found in *Rhinolophus* sp. and *Hipposideros* sp. It should be noted that the SCR phylogeny was constructed on a short sequence length of 167nt (RMH used 410nt) following MSA trimming to include maximum

sequences for phylogenetic tree construction. The drawback of this is the reduced number of characters available for taxa sampling, which play a significant role in determining the substitution model for molecular evolution, and its subsequent use in bayesian phylogenetic tree construction, support and its inference (Blackburne & Whelan, 2013; Shen et al., 2016; Wortley et al., 2005). Simon (2022) and Wortley et al.,(2005) expound on how improved sequence length subsequently improves branch support, where low support values at internal nodes result in uncertainty in establishing phylogenetic relationship amongst sequences. Therefore, the **PMV** sequences detected using the SCR and the subsequent phylogenetic tree provide limited but expected insight to the evolutionary relationship between detected sequences and previously reported **PMVs**. Further analysis using a wider target-region assay is required to verify the evolutionary relationship.

The detection of similar CoV and **PMV** sequences in this study with those previously reported in South Africa, Zimbabwe, Cameroon, Rwanda, Ghana, and DRC highlights circulation of related viruses within the SADC and Sub-Saharan region. This may be due to various contributing factors. As previously discussed, bat migration is one factor that has made bats successful viral natural host. Studies in bat migration patterns are limited with most documented migration patterns observed and reported in Europe and North America (Fleming, 2019). The introduction, maintenance, and circulation of these viruses may be due to the interaction between bats of similar species in Botswana and its neighbors through migration. Though there is limited information on the migration patterns of individual bat families, **certain** cave-dwelling bats, **such as horseshoe bats**, are thought to be nonmigratory, or travel shorter distances (Hutterer et al., 2005). We cannot rule out that there may be **the** introduction of other **cave-dwelling** bat species **that are migratory, such as *Miniopterus* bats**, in addition to the three **suspected bat species**, as extensive bat species richness and migration patterns on the bats of Gcwihaba **have** not been carried out. It could be that there may be other bat species that exhibit longer distance migration patterns due to seasonal change in the “base” habitat so they periodically use the cave as a habitat (Krauel & McCracken, 2013; Popa-Lisseanu & Voigt, 2009).

Despite the geographical difference, another factor that may account for similarity in partial viral sequences to regionally reported CoV and **PMV** viruses, is the roosting behavior of bats. Caves create stable environments that support more than one bat species (Furey & Racey, 2016), and have various structural layouts that meet specific bat species habitat requirements. Through direct interactions, transmission across bat families may be possible within a cave roost, particularly when there is direct contact. This could explain why all three bat families mentioned within the study were found to harbor **PMV** RNA, i.e., a consequence of virus

sharing among the three species due to shared roost. Lastly, Streicker et al., (2010) discusses how bat species with similar innate immunity evolution may facilitate viral sharing. The two bats; horseshoe bat (*Rhinolophus*) and leaf-nosed bats (*Hipposideros*) are phylogenetically related (superfamily: *Rhinolophoidea*) (Amador et al., 2018) thus may subsequently have similar host defenses, which may reduce the hurdle of viral adaption within the two genera.

In summary, viral RNA of coronaviruses and paramyxoviruses within fecal samples of bats inhabiting Gcwihaba caves was detected using amplification of partial conserved regions, RdRp and L gene, successfully. Accompanied with the various factors that have made bats successful natural hosts of viral pathogens, there are implications that similar viruses may be present within bat hosts that are found within close proximity to human settlements. This stresses the need to carry out further studies within areas where bat-human interaction is increased such as semi-urban areas. The study findings also underscore the need for proper bat species association with viral pathogens to advance the understanding of bat-virus dynamics within Botswana and the SADC region.

Chapter 5: Conclusion

The detection of the presence of coronaviruses and paramyxoviruses within the bats inhabiting Gcwihaba caves is the first of its kind in Botswana. The study has provided preliminary data for the presence of these **viruses in cave-dwelling bats, which are known to be reservoirs for potential** zoonotic pathogens. It is worth noting that paramyxovirus viral sharing was observed between *Rhinolophus* and *Hipposideros* bats, suggesting that these viruses might have a broader host range. This is a critical consideration, as viruses with a wider host range pose a risk of spillover to other mammalian species such as humans and livestock. **The vulnerability of tourists and cave guides to events of spillover of the viral pathogens cannot be undermined.** Literature has indicated that various factors are taken into consideration on the success of animal associated viral pathogens to switch over to human hosts. These factors include efficient receptor usage by viral pathogens, interactions with host factors, successful innate immune evasion. To date, understanding on spillover of paramyxoviruses shows evidence on the importance of an intermediate host and subsequent interaction of these intermediate hosts with the human population. This highlights how different viral pathogens with zoonotic potential follow distinct pathways during spillover events. Due to limited understanding on these pathways, it is essential that preventative measures are taken to mitigate potential spillovers.

Study limitations

Although the current study provides invaluable preliminary data on occurrence of certain viruses within a selected bats population in Botswana, it is important to note the inherent shortcomings of the study. Firstly, the methods used in the detection require revision particularly in terms of sample methodology. Sample types used for PMV surveillance needs to be changed from fecal to urine samples as urine samples are more sensitive in terms of viral detection. Furthermore, fecal samples are more prone to cross contamination by urine during under-roost sampling which may affect accuracy of viral detection. Though the under-roost sampling method provides an indirect measure of viral positive proportions, it may not truly capture the true prevalence of coronaviruses and paramyxoviruses within the bat population in Gcwihaba caves. The time allowed between setup of sampling sheets needs to be reduced to reduce urine contamination. Moreover, the noninvasive method of putting sheets under select “mini roosts” requires readjustment in terms of plastic sheet size, number and placement in order to optimize sampling efficacy. This noninvasive sampling technique is a direction that may be explored especially in performing longitudinal surveillance studies for

understanding the variation of viral prevalence and richness at different seasons. This is particularly beneficial in developing countries where bat capturing and handling expertise and the presence of expensive equipment is relatively low.

Species identification was limited to visual inspection of morphological characteristics, this became a hurdle as the horseshoe bats (genus *Rhinolophus*) were found to carry *Merbecovirus* partial viral VRNA, a phenomenon which is uncommon though not unlikely due to their flexible host specificity. The *Rhinolophus* genus is typically associated with the SARS and SARS-like coronaviruses, and merbecoviruses are associated with other bat host species, but have previously been reported in similar species in Zimbabwe.

Additionally, in the detection of paramyxoviruses, the phylogenetic relationship determined using the RMH and SCR assay indicated closely related viruses to those identified in South Africa, Gabon, Rwanda, Cameroon according to bat species. However, the SCR assay had limitations in phylogeny inference as the subsequent phylogenetic relationship was limited but did give a scope of related viruses. Further molecular assay revision is required to increase the target region amplification product length so have better insight on the phylogenetic relationship.

Given the detection of viruses in both the wet and dry seasons, with CoV being higher in the wet season and PMV higher in the dry season, it is advisable to recommend year-round precautions to lower the risk of viral exposure. These precautions, which include wearing personal protective equipment such as gloves and masks and implementing comprehensive hygiene measures such as hand washing, may facilitate mitigating the risk of viral exposure during cave exploration by tourists and cave guides. Furthermore, genetic identification of bat species associated with specific viruses should be carried out alongside any further studies on viral diversity. Whole genome sequence (WGS) characterization can shed more light on how closely the detected viral RNA matches previously identified viruses, especially when partial genomic studies suggest potential novelty. In countries like Botswana, using WGS to identify viruses and compare them to existing strains can be challenging due to cost constraints. However, targeting gene sequencing or comparative analysis of specific genes can be a more practical and cost-effective approach to identifying viral species and their genetic relationships in most developing countries. Lastly, the seasonal viral prevalence is greatly limited and requires longitudinal sampling to get a comprehensive outlook on the prevalence of viral pathogens across seasons in Botswana.

Future directions

The data presented shows the necessity of carrying out further viral studies in bats in Botswana, particularly where there are higher human-bat interactions such as in public areas. Public domains present higher risk interfaces for potential spillover of zoonotic diseases, which is a significant public health concern. To gain a deeper understanding of viral diversity and to overcome the limitations of primer specificity in conventional PCR (or RT-PCR), we recommend conducting explorative studies, including metagenomic studies with deep genome sequencing.

It is crucial to consider the potential presence of other viruses of public health concern that are associated with bats such as human coronaviruses HCoV-NL63 and HCoV-229E, as well as other DNA viruses that may go undetected. Furthermore, the study of other hosts, particularly rodents, may also help gain insight into the surveillance of viral zoonotic pathogens. Rodents, alongside bats, are reportedly successful natural hosts for zoonotic pathogens, and understating their interactions, especially shared geographical areas, is vital to understanding viral sharing between the two hosts. To mitigate the potential of spillover of zoonotic pathogens, it is essential to investigate viral zoonotic pathogens within rodent hosts, not only in public areas but also in wildlife zones such as national parks. Botswana's reliance on tourism underscores the need for a comprehensive understanding of viral zoonotic diversity and ecology for disease management.

Information sharing on zoonotic diseases with the general public is also a possible preemptive measure that should be taken. Collaborating with relevant stakeholders such as the Ministry of Health & Wellness, and the Ministry of Environment, Wildlife & Tourism, can help create a framework for information dissemination. The SARS-CoV-2 pandemic has provided an opportunity to educate the public on zoonotic diseases, their hosts, and how to coexist safely with wildlife implicated as **natural** hosts without resorting to their extermination.

Chapter 6: Bibliography

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Chapter 7: Appendix

Table 7.1: Sample screening details

Season of Collection *	Family	Genus	Field ID	Other ID	Coronavirus	RMH	SCR	SCR Clone	CoV Phylogeny	PMV Phylogeny
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B001	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B002	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B003	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B004	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B005	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B006	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B007	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B008	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B009	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B010	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B011	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B012	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B013	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW004	DON005B014	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B001	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B002	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B003	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B004	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B005	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B006	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B007	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B008	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B009	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B010	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B011	Neg	Neg	Neg	N/A	N/A	N/A

Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B012	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B013	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B014	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B015	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B016	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B017	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B018	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B019	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B020	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B021	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B022	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B023	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B024	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B025	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B026	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B027	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B028	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B029	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B030	Neg	Neg	ü	N/A	N/A	Jeilongvirus
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B031	Neg	Neg	Pos	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B032	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B033	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Rhinolophidae	Rhinolophus	GCW005	DON006B034	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B001	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B002	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B003	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B004	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B005	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B006	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B007	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B008	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B009	Neg	Neg	Pos	û	N/A	Jeilongvirus-related

Dry	Nycteridae	Nycteris	GCW006	DON007B010	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B011	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B012	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B013	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B014	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B015	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B016	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B017	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B018	Neg	Neg	Pos	ü	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B019	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B020	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B021	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B022	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B023	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B024	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B025	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B026	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B027	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B028	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B029	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B030	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B031	Neg	Neg	Pos	ü	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B032	Neg	Neg	Neg	ü	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B033	Neg	Pos	Neg	ü	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B034	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B035	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B036	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B037	Neg	Neg	Pos	ü	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B038	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B039	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B040	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B041	Neg	Neg	Neg	N/A	N/A	N/A

Dry	Nycteridae	Nycteris	GCW006	DON007B042	Neg	Neg	Pos	ü	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B043	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B044	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B045	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B046	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B047	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Dry	Nycteridae	Nycteris	GCW006	DON007B048	Neg	Neg	Pos	ü	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B049	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Dry	Nycteridae	Nycteris	GCW006	DON007B050	Neg	Neg	Neg	N/A	N/A	N/A
Dry	Nycteridae	Nycteris	GCW006	DON007B051	Neg	Neg	Pos	û	N/A	Jeilongvirus-related
Wet	Rhinolophidae	Rhinolophus	GCW008_8	DON008B001	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW008_8	DON008B002	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW008_8	DON008B003	Pos	Neg	Neg	N/A	Merbecovirus	N/A
Wet	Rhinolophidae	Rhinolophus	GCW008_8	DON008B004	Pos	Neg	Neg	N/A	Merbecovirus	N/A
Wet	Rhinolophidae	Rhinolophus	GCW008_8	DON008B005	Pos	Neg	Neg	N/A	Merbecovirus	N/A
Wet	Rhinolophidae	Rhinolophus	GCW008_8	DON008B006	Pos	Neg	Neg	N/A	Merbecovirus	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B001	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B002	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B003	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B004	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B005	Neg	Neg	Pos	û	N/A	Jeilongvirus
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B006	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B007	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B008	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B009	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B010	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B011	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B012	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B013	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B014	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B015	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B016	Neg	Neg	Neg	N/A	N/A	N/A

Wet	Macronycteris	Hipposideros	GCW008_9	DON009B017	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B018	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B019	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B020	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B021	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B022	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B023	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B024	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B025	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B026	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Macronycteris	Hipposideros	GCW008_9	DON009B027	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B001	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B002	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B003	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B004	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B005	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B006	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B007	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B008	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B009	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B010	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B011	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B012	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B013	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B014	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B015	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B016	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B017	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B018	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B019	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B020	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B021	Neg	Neg	Neg	N/A	N/A	N/A

Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B022	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B023	Neg	Neg	Pos	û	N/A	Jeilongvirus
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B024	Neg	Pos	Neg	N/A	N/A	Jeilongvirus
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B025	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B026	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B027	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B028	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B029	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B030	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B031	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B032	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B033	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B034	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B035	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B036	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B037	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B038	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B039	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B040	Neg	Neg	Neg	N/A	N/A	N/A
Wet	Rhinolophidae	Rhinolophus	GCW009	DON010B041	Neg	Neg	Neg	N/A	N/A	N/A

(*) The season of collection “Dry” corresponds to June 2020 (no rain), while “Wet” corresponds to collection done in March 2021 (rainy season in Botswana)

Neg -negative; Pos-positive; N/A- not applicable; (û) two clone sequences available; (û) one sequence available from assay.

Table 7.2: Sequences included in phylogenetic analysis of Coronavirus partial RdRp gene

GenBank Accession	Virus ID	Host	Location
AC000192	Murine hepatitis virus	‡	‡
DQ071615	Bat SARS coronavirus/Rp3/CHINA	Bat	‡
DQ412043	SARS CoV/BatCoV/Rhinolophus/Rm1/2004	Bat (<i>Rhinolophus macrotis</i>)	‡
EF065513	Rousettus BatCoV/HKU9	Bat	China: Guangdong
EF065515	BatCoV/HKU9-3	Bat	China: Guangdong
EF065516	BatCoV/ HKU9-4	Bat	China: Guangdong
EF446615	Equine coronavirus strain NC99	lab host (HRT-18G cells)	‡
FJ710054	BatCoV/Hipposideros/GhanaKwam/22/2008	Bat (<i>Hipposideros</i> sp.)	Ghana

GU190215	BatCoV/Rhinolophus/BM48-31/BGR/2008	Bat (<i>Rhinolophus blasii</i>)	Bulgaria
HM211098	BatCoV/ HKU9-5-1	Bat	China: Guangdong
HM211099	BatCoV/ HKU9-5-2	Bat	China: Guangdong
HM211100	BatCoV/HKU9-10-1	Bat	China: Guangdong
HQ728481	BatCoV/Chaerephon/Kenya/KY41/2006	Bat (<i>Chaerephon pumilus</i>)	Kenya
HQ728485	BatCoV/Miniopterus/Kenya/KY33/2006	Bat (<i>Miniopterus inflatus</i>)	Kenya
JQ649535	BatCoV/Rhinolophus/Rwanda/441/2008	Bat (<i>Rhinolophus clivosus</i>)	Rwanda
JQ649536	BatCoV/Rhinolophus/Rwanda/445/2008	Bat (<i>Rhinolophus clivosus</i>)	Rwanda
JX174638	BatCoV/Hipposideros/Gabon/292/2009	Bat (<i>Hipposideros caffer</i>)	Gabon
JX899383	BatCoV/Nycteris/KW2E-F93/GHANA/2010	Bat (<i>Nycteris</i> sp.)	Ghana
KC633198	BatCoV/Rhinolophus/It1/ITA/2009	Bat (<i>Rhinolophus ferrumequinum</i>)	Italy
KC633199	BatCoV/Rhinolophus/It17/ITA/2009	Bat (<i>Rhinolophus ferrumequinum</i>)	Italy
KC633200	BatCoV/Rhinolophus/It2/ITA/2009	Bat (<i>Rhinolophus ferrumequinum</i>)	Italy
KC633201	BatCoV/Rhinolophus/BB89-22/BGR/2008/1-1652	Bat (<i>Rhinolophus blasii</i>)	Bulgaria
KC633202	BatCoV/Rhinolophus/BB99-04/BGR/2009	Bat (<i>Rhinolophus euryale</i>)	Bulgaria
KC633203	BatCoV/Rhinolophus/BB89-98/BGR/2008	Bat (<i>Rhinolophus euryale</i>)	Bulgaria
KC633204	BatCoV/Rhinolophus/BB89-92/BGR/2008	Bat (<i>Rhinolophus euryale</i>)	Bulgaria
KC633206	BatCoV/Rhinolophus/It15/ITA/2009	Bat (<i>Rhinolophus ferrumequinum</i>)	Italy
KC633207	BatCoV/Rhinolophus/FR0711-B3/FRA/2011	Bat (<i>Rhinolophus ferrumequinum</i>)	France
KC869678	BatCoV/Neoromicia/PML-PHE1/RSA/2011	Bat (<i>Neoromicia capensis</i>)	South Africa
KF367457	Bat SARS-like/WIV1/Rhinolophus/CHINA	Bat (<i>Rhinolophus sinicus</i>)	China
KF843853	BatCoV/Mops/BtCoV/NCL_MCO1/RSA/2012	Bat (<i>Mops cf. condylurus</i>)	South Africa
KF843858	BatCoV/Neoromicia/GrNC4/Neo_cap/RSA/2012	Bat (<i>Neoromicia cf. capensis</i>)	South Africa
KF859759	BatCoV/BEM003F/4771-10	Bat (<i>Pteropus rufus</i>)	Madagascar: Bemanonga
KF859762	BatCoV/ NK045F/ 4909-10	Bat (<i>Pteropus rufus</i>)	Madagascar: Ankiliabo
KF859767	BatCoV/BEM070F/8550-11	Bat (<i>Pteropus rufus</i>)	Madagascar: Bemanonga
KF917527	CamelCoV/MERS-CoV/SAUDI ARABIA	Camel	Saudi Arabia

KF958702	HCoV/MERS-CoV/SAUDI ARABIA	Human (<i>Homo sapiens</i>)	Saudi Arabia
KJ713297	CamelCoV/MERS-CoV/503/SAUDI ARABIA	Camel (<i>Camelus dromedarius</i>)	Saudi Arabia
KJ713299	CamelCoV/MERS-CoV/376/SAUDI ARABIA	Camel (<i>Camelus dromedarius</i>)	Saudi Arabia
KJ866065	HCoV/HKU1/CU-A396/2013/THAILAND	Human (<i>Homo sapiens</i>)	Thailand
KM015348	HCoV/MERS-CoV/England/2/2013	Human (<i>Homo sapiens</i>)	England
KM349744	RatCoV/Rattus/HKU24-R05010I	Rat (<i>Rattus norvegicus</i>)	China
KP112152	HCoV/HKU1/KENYA/008/2009	Human (<i>Homo sapiens</i>)	Kenya
KT346237	BatCoV/Rousettus/Egypt/NRC-HKU-034	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KT346238	BatCoV/Rousettus/Egypt/NRC-HKU-092	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KT346239	BatCoV/Rousettus/Egypt/NRC-HKU-108	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KT346240	BatCoV/ Rousettus/Egypt/NRC-HKU-302	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KT346241	BatCoV/Rousettus/Egypt/NRC-HKU-306	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KT346242	BatCoV/Rousettus/Egypt/NRC-HKU-308	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KT346243	BatCoV/Rousettus/Egypt/NRC-HKU-310	Bat (<i>Rousettus aegyptiacus</i>)	Egypt
KU308549	MERS-related HCoV/Korea/Seoul/SNU1-035/2015	Human (<i>Homo sapiens</i>)	South Korea
KU745562	HCoV/OC43/ 4467A/2015/MEXICO	Human (<i>Homo sapiens</i>)	China: Beijing
KX284981	BatCoV/Hipposideros/PREDICT_CoV-32/GVF-CM-ECO70036/CAMEROON	Bat (<i>Hipposideros caffer</i>)	Cameroon
KX285111	BatCoV/Rhinolophus/PREDICT CoV-42 AABSA/RWANDA	Bat (<i>Rhinolophus</i> sp.)	Rwanda
KX285250	RodentCoV/Mastomys/PREDICT_CoV-64/AARZQ	Mouse (<i>Mastomys</i> sp.)	Tanzania
KX285253	BatCov/PREDICT_CoV-62/AATKH/TANZANIA	Bat	Tanzania
KX344031	HCoV/OC43/LRTI_238	Human (<i>Homo sapiens</i>)	Mexico
KX574227	BatCoV/Pipistrellus/PREDICT/PDF-2180/UGANDA	Bat (<i>Pipistrellus cf. hesperidus</i>)	Uganda
KY073745	NL63-related BatCoV/Triaenops/BtKYNL63-9b/KENYA	Bat (<i>Triaenops afer</i>)	Kenya
KY073746	NL63-related BatCoV/Triaenops/BtKYNL63-15/KENYA	Bat (<i>Triaenops afer</i>)	Kenya
KY352407	SARS-related BatCoV/Rhinolophus/KENYA/BtKY72	Bat (<i>Rhinolophus</i> sp.)	Kenya
KY370052	RodentCoV/Meriones/RtMm-CoV-1/IM2014 CHINA	Midday jird (<i>Meriones meridianus</i>)	China
KY370072	RodentCoV/Apodemus/RtAd-CoV/SX2014 CHINA	South China field Mouse (<i>Apodemus draco</i>)	China
KY370076	RodentCoV/Rattus/RtRf-CoV/GX2016 CHINA	Rat (<i>Rattus flavipectus</i>)	China

MF593268	MERS-related BatCoV/Neoromicia/5038/RSA	Bat (<i>Neoromicia capensis</i>)	South Africa
MG000865	229E-related BatCoV/Hipposideros/BtCov-Zim001Mab	Bat (<i>Hipposideros</i> sp.)	Zimbabwe
MG000872	Bat SARS-like/BtCov-Zim035Mag	Bat (<i>Hipposideros</i> sp.)	Zimbabwe
MG963186	BatCoV/Hipposideros/GABON/13GB0214	Bat (<i>Hipposideros</i> sp.)	Zimbabwe
MG963187	BatCoV/Hipposideros/GABON/13GB0215	Bat (<i>Hipposideros gigas</i>)	Gabon
MG963188	BatCoV/Hipposideros/GABON/13GB0273	Bat (<i>Hipposideros gigas</i>)	Gabon
MG963198	BatCoV/Hipposideros/09GB0761/GABON	Bat (<i>Hipposideros cf ruber</i>)	Gabon
MH170089	BatCoV/Hipposideros/622/KENYA	Bat (<i>Hipposideros caffer</i>)	Kenya
MH170126	BatCoV/Hipposideros/2368/KENYA	Bat (<i>Hipposideros caffer</i>)	Kenya
MH170128	BatCoV/Rhinolophus/2621/KENYA	Bat (<i>Rhinolophus</i> sp.)	Kenya
MK211376	BatCoV/Rhinolophus/BtRs-BetaCoV/YN2018B	Bat (<i>Rhinolophus affinis</i>)	China
MK569432	BatCoV/Miniopterus/UP7530/GP/RSA/2017	Bat (<i>Miniopterus natalensis</i>)	South Africa
MK569443	BatCoV/Miniopterus/UP8489/LP/RSA/2017	Bat (<i>Miniopterus natalensis</i>)	South Africa
MN026164	HCoV/OC43/KLF/01/2018/KENYA	Human (<i>Homo sapiens</i>)	Kenya
MN183161	BatCoV/Miniopterus/19006/MOZAMBIQUE	Bat (<i>Miniopterus mossambicus</i>)	Mozambique: Inhassoro
MN183171	BatCoV/Hipposideros/18951/MOZAMBIQUE	Bat (<i>Hipposideros caffer</i>)	Mozambique: Inhassoro
MN183189	BatCoV/Rhinolophus/19029/MOZAMBIQUE	Bat (<i>Rhinolophus lobatus</i>)	Mozambique: Inhassoro
MN183190	BatCoV/Rhinolophus/19038/MOZAMBIQUE	Bat (<i>Rhinolophus rhodesiae</i>)	Mozambique: Inhassoro
MN183193	BatCoV/Nycteris/18971/MOZAMBIQUE	Bat (<i>Nycteris thebaica</i>)	Mozambique: Inhassoro
MN183194	BatCoV/18970/MOZAMBIQUE	Bat (<i>Nycteris thebaica</i>)	Mozambique: Inhassoro
MN183195	BatCoV/Nycteris/18974/MOZAMBIQUE	Bat (<i>Nycteris thebaica</i>)	Mozambique: Inhassoro
MN183196	BatCoV/18972/Nycteris/MOZAMBIQUE	Bat (<i>Nycteris thebaica</i>)	Mozambique: Inhassoro
MN514966	CamelCoV/Dromedary/HKU23/Nigeria/NV1097/2015	Camel (<i>Camelus dromedarius</i>)	Nigeria
MN988669	HCoV/SARS-2/2019-nCoV/WHU02	Human (<i>Homo sapiens</i>)	China
MN996532	BatCoV/Rhinolophus/RaTG13	Bat (<i>Rhinolophus affinis</i>)	China
MT040336	PangolinCoV/CoV_GX-P5E/CHINA	Malayan pangolin (<i>Manis javanica</i>)	China
MT084071	PangolinCoV/MP789/CHINA	Malayan pangolin (<i>Manis javanica</i>)	China
MT135044	SARS-CoV-2/human/CHN/235/2020	Human (<i>Homo sapiens</i>)	China: Beijing
MT163721	SARS-CoV-2/human/USA/WA9-UW6/2020	Human (<i>Homo sapiens</i>)	USA: WA
MT586840	BatCoV/Hipposideros/GUINEA/CCGU86	Bat (<i>Hipposideros ruber</i>)	Guinea: Kindia

MT586851	BatCoV/Nycteris/CCGU235/GUINEA	Bat (<i>Hipposideros ruber</i>)	Guinea: Kindia
MT586852	BatCoV/Hipposideros/CCGU269/GUINEA	Bat (<i>Hipposideros ruber</i>)	Guinea: Kankan
NC_002645	HCoV 229E	‡	‡
NC_004718	SARS coronavirus Tor2	Human (<i>Homo sapiens</i>)	Canada: Toronto
NC_006577	Human coronavirus HKU1	Human (<i>Homo sapiens</i>)	‡
NC_009020	BatCoV/Pipistrellus/HKU5/CHINA	Bat	China: Guangdong
NC_009988	Rhinolophus bat coronavirus HKU2 CHINA	Bat	‡
NC_010438	Miniopterus bat coronavirus HKU8	Bat	Hong Kong
NC_018871	Rousettus bat coronavirus HKU10	Bat	China
NC_025217	BatCoV/Hipposideros/CHINA	Bat (<i>Hipposideros pratti</i>)	China
NC_028811	BatCoV/Myotis/BtMr-AlphaCoV/SAX2011	Bat (<i>Myotis ricketti</i>)	China
NC_030886	Rousettus bat coronavirus GCCDC1 356	Bat (<i>Rousettus leschenaultii</i>)	China
NC_032107	NL63-related BatCoV/Triaenops/ BtKYNL63-9a/KENYA	Bat (<i>Triaenops afer</i>)	Kenya
OM469983	BatCoV/CHI-143/ZIMBABWE	Bat	Zimbabwe
OM469987	BatCoV/Nycteris/CHI-843/ZIMBABWE	Bat (<i>Nycteris</i> sp.)	Zimbabwe
OM469984	BatCoV/Nycteris/CHI-835/ZWE/2020	Bat (<i>Nycteris</i> sp.)	Zimbabwe
OM469985	BatCoV/CHI-851/ ZWE/2020	Bat	Zimbabwe
OM470176	BatCoV/Hipposideros/MAG-1150/ZWE/2021	Bat (<i>Hipposideros</i> sp.)	Zimbabwe
OM470177	BatCoV/Rhinolophus/MAG-1094/2021	Bat (<i>Rhinolophus</i> sp.)	Zimbabwe

* information not listed

Table 7.3: Uncorrected p- distance for the evolutionary divergence between selected sequences of the partial RdRp gene

The number of base differences per site from between sequences are shown as percentages. Standard error estimate(s) are shown above the diagonal in blue and were obtained by a bootstrap procedure (1000 replicates). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.88). This analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were 207 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

	Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	NC_030886 Rousettus bat coronavirus GCCDC1 356		2.96	3.35	3.38	3.51	3.50	3.50	3.50	3.50	3.51	3.50	3.50	3.49	3.49	3.49	3.49	3.38	3.39	3.49	3.47	3.40	3.35	3.43	3.25
2	HM211099 BatCoV/ HKU9-5-2	25.1 2		3.37	3.34	3.48	3.46	3.46	3.46	3.49	3.47	3.48	3.49	3.44	3.47	3.44	3.44	3.29	3.29	3.46	3.48	3.32	3.31	3.27	3.29
3	KP112152 HCoV/HKU1/008/KEN/2009	36.7 1	35.7 5		2.85	3.47	3.54	3.54	3.54	3.53	3.54	3.50	3.49	3.50	3.50	3.50	3.50	3.39	3.43	3.42	3.26	3.50	3.38	3.43	3.45
4	MN026164 HCoV/OC43/KLF/01//KEN/2018	38.6 5	35.2 7	21.2 6		3.50	3.54	3.54	3.54	3.56	3.55	3.48	3.48	3.54	3.51	3.54	3.54	3.48	3.52	3.52	3.35	3.48	3.47	3.55	3.49
5	OM469983 BatCoV/CHI-143/ZWE/2020	44.9 3	39.1 3	33.8 2	37.6 8		1.19	1.19	1.19	1.42	1.29	0.98	0.84	1.04	0.68	1.04	1.04	3.55	3.55	3.49	3.43	3.43	3.51	3.60	3.41

	Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
6	OM469984 BatCoV/Nycteris/CHI-835/ZWE/2020	43.9 6	39.1 3	35.7 5	40.1 0	2.90		0.00	0.00	0.80	0.46	1.21	1.09	1.10	1.34	1.10	1.10	3.51	3.51	3.48	3.46	3.43	3.50	3.59	3.4 4
7	OM469985 BatCoV/CHI-851/ZWE/2020	43.9 6	39.1 3	35.7 5	40.1 0	2.90	0.00		0.00	0.80	0.46	1.21	1.09	1.10	1.34	1.10	1.10	3.51	3.51	3.48	3.46	3.43	3.50	3.59	3.4 4
8	OM469987 BatCoV/Nycteris/CHI-843/ZWE/2020	43.9 6	39.1 3	35.7 5	40.1 0	2.90	0.00	0.00		0.80	0.46	1.21	1.09	1.10	1.34	1.10	1.10	3.51	3.51	3.48	3.46	3.43	3.50	3.59	3.4 4
9	OM470176 BatCoV/Hipposideros/MAG-1150/ZWE/2021	44.9 3	40.1 0	36.7 1	40.5 8	4.35	1.45	1.45	1.45		0.92	1.40	1.31	1.35	1.54	1.35	1.35	3.55	3.53	3.48	3.46	3.47	3.49	3.57	3.4 4
10	OM470177 BatCoV/Rhinolophus/MAG-1094/2021	44.4 4	39.6 1	36.2 3	40.5 8	3.38	0.48	0.48	0.48	1.93		1.29	1.19	1.19	1.43	1.19	1.19	3.51	3.52	3.48	3.45	3.43	3.50	3.60	3.4 4
11	MK569432 BatCoV/Miniopterus/UP7530/GP/RSA/2017	44.4 4	40.5 8	35.7 5	38.1 6	1.93	2.90	2.90	2.90	4.35	3.38		0.51	1.46	1.18	1.46	1.46	3.56	3.56	3.47	3.48	3.44	3.50	3.60	3.4 4
12	MK569443 BatCoV/Miniopterus/UP8489/LP/RSA/2017	44.4 4	40.1 0	35.2 7	38.1 6	1.45	2.42	2.42	2.42	3.86	2.90	0.48		1.36	1.08	1.36	1.36	3.56	3.56	3.47	3.45	3.44	3.51	3.60	3.4 3
13	BatCoV/Rhinolophus/DON008B003/BW/2021	44.4 4	38.1 6	34.3 0	39.1 3	2.42	2.42	2.42	2.42	3.86	2.90	4.35	3.86		1.22	0.00	0.00	3.52	3.51	3.52	3.45	3.45	3.51	3.58	3.4 2
14	BatCoV/Rhinolophus/DON008B004/BW/2021	45.4 1	38.6 5	34.7 8	38.1 6	0.97	3.86	3.86	3.86	5.31	4.35	2.90	2.42	3.38		1.22	1.22	3.56	3.56	3.48	3.44	3.41	3.51	3.60	3.4 1
15	BatCoV/Rhinolophus/DON008B005/BW/2021	44.4 4	38.1 6	34.3 0	39.1 3	2.42	2.42	2.42	2.42	3.86	2.90	4.35	3.86	0.00	3.38		0.00	3.52	3.51	3.52	3.45	3.45	3.51	3.58	3.4 2
16	BatCoV/Rhinolophus/DON008B006/BW/2021	44.4 4	38.1 6	34.3 0	39.1 3	2.42	2.42	2.42	2.42	3.86	2.90	4.35	3.86	0.00	3.38	0.00		3.52	3.51	3.52	3.45	3.45	3.51	3.58	3.4 2
17	KY352407 SARS-related BatCoV/Rhinolophus/BtKY72/KEN/2007	39.6 1	32.3 7	37.2 0	41.0 6	38.6 5	38.6 5	38.6 5	38.6 5	39.6 1	39.1 3	39.6 1	39.6 1	37.6 8	39.1 3	37.6 8	37.6 8		1.18	3.35	3.33	3.44	3.34	3.29	3.4 9
18	JQ649536_2 BatCoV/Rhinolophus/Rwanda/445/2008	40.1 0	33.8 2	37.6 8	41.5 5	37.6 8	37.6 8	37.6 8	37.6 8	38.6 5	38.1 6	38.6 5	38.6 5	36.7 1	38.1 6	36.7 1	36.7 1	2.90		3.32	3.30	3.41	3.36	3.26	3.4 4
19	MH170126 BatCoV/Hipposideros/2368/KEN/2015	41.5 5	36.2 3	35.2 7	38.1 6	39.1 3	40.5 8	40.5 8	40.5 8	41.5 5	41.0 6	40.1 0	40.1 0	40.5 8	38.6 5	40.5 8	40.5 8	33.8 2	34.3 0		2.65	3.47	3.47	3.41	3.3 5
20	MG000872 Bat SARS-like/BtCov-Zim035Mag/ZWE/2016	42.5 1	41.0 6	33.3 3	36.7 1	38.1 6	37.6 8	37.6 8	37.6 8	38.6 5	38.1 6	38.1 6	37.6 8	39.1 3	38.6 5	39.1 3	39.1 3	35.2 7	36.2 3	18.3 6		3.45	3.36	3.47	3.3 1
21	NC_018871 Roussetus bat coronavirus HKU10	44.9 3	43.4 8	44.9 3	47.8 3	43.9 6	44.9 3	44.9 3	44.9 3	44.4 4	45.4 1	45.4 1	44.9 3	44.9 3	44.4 4	44.9 3	44.9 3	45.8 9	45.4 1	45.8 9	44.9 3		3.37	3.18	3.3 0
22	NC_028811 BatCoV/Myotis/BtMr-AlphaCoV/SAX2011	38.6 5	37.6 8	41.5 5	43.9 6	51.2 1	50.2 4	50.2 4	50.2 4	50.7 2	50.7 2	51.6 9	51.2 1	49.7 6	51.2 1	49.7 6	49.7 6	40.5 8	41.0 6	43.4 8	43.4 8	32.8 5		3.02	3.1 1
23	KF843858 BatCoV/Neoromicia/GrNC4/RSA/2012	42.0 3	39.1 3	41.0 6	43.0 0	45.8 9	45.8 9	45.8 9	45.8 9	46.8 6	46.3 8	46.8 6	46.3 8	44.4 4	45.8 9	44.4 4	44.4 4	38.1 6	37.6 8	43.4 8	39.6 1	28.5 0	25.1 2		2.9 5
24	HQ728481 BatCoV/Chaerephon/KEN/KY41/2006	41.5 5	36.2 3	36.2 3	42.0 3	40.5 8	41.5 5	41.5 5	41.5 5	43.0 0	42.0 3	42.5 1	42.0 3	40.5 8	40.5 8	40.5 8	40.5 8	39.6 1	39.6 1	35.2 7	37.2 0	32.3 7	28.0 2	25.6 0	

Sequence identities are as follows: NC_030886 Roussetus bat coronavirus GCCDC1 356, HM211099 BatCoV/ HKU9-5-2, KP112152 HCoV/HKU1/008/KEN/2009, MN026164 HCoV/OC43/KLF/01/KEN/2018, OM469983 BatCoV/CHI-143/ZWE/2020, OM469984 BatCoV/Nycteris/CHI-835/ZWE/2020, OM469985 BatCoV/CHI-851/ZWE/2020, OM469987 BatCoV/Nycteris/CHI-843/ZWE/2020, OM470176 BatCoV/Hipposideros/MAG-1150/ZWE/2021, OM470177 BatCoV/Rhinolophus/MAG-1094/2021, MK569432 BatCoV/Miniopterus/UP7530/GP/RSA/2017, MK569443 BatCoV/Miniopterus/UP8489/LP/RSA/2017, BatCoV/Rhinolophus/DON008B003/BW/2021, BatCoV/Rhinolophus/DON008B004/BW/2021, BatCoV/Rhinolophus/DON008B005/BW/2021, BatCoV/Rhinolophus/DON008B006/BW/2021, KY352407 SARS-related BatCoV/Rhinolophus/BtKY72/KEN/2007, JQ649536_2 BatCoV/Rhinolophus/RWA/445/2008, MH170126 BatCoV/Hipposideros/2368/KEN/2015, MG000872 Bat SARS-like/BtCov-Zim035Mag/ZWE/2016, NC_018871 Roussetus bat coronavirus HKU10, NC_028811 BatCoV/Myotis/BtMr-AlphaCoV/SAX2011, KF843858 BatCoV/Neoromicia/GrNC4/RSA/2012, and HQ728481 BatCoV/Chaerephon/KEN/KY41/2006

Table 7.4: Sequences included in Paramyxovirus phylogeny

GenBank Accession	Virus Identity	Host	Location
AB016162	Measles virus	‡	‡
AF128244	Measles virus Gambia	‡	Gambia
AF178654	Bovine parainfluenza virus 3	‡	‡
AF266291	Measles virus strain	‡	‡
AJ608288	Dolphin morbillivirus	‡	‡
AJ849636	Peste des petits ruminants virus	Sheep (<i>Ovis aries</i>)	Türkiye
AY988601	Nipah virus/BAN	Human (<i>Homo sapien</i>)	Bangladesh
DQ100461	Beilong virus	‡	‡
EU439429	Swine parainfluenza virus 3	Pig	‡
FJ609191	BatPV/Eidolon/GH10/GHA/2008	Bat (<i>Eidolon helvum</i>)	Ghana
FJ609194	BatPV/Eidolon/GH48/GHA/2008	Bat (<i>Eidolon helvum</i>)	Ghana
FJ971940	BatPV/Eidolon/GH27/GHA/2009	Bat (<i>Eidolon helvum</i>)	Ghana
FN869553	Nipah virus	Bat (<i>Pteropus vampyrus</i>)	Malaysia
GQ168929	BatPV/Eid.hel/GH45/GHA2008	Bat (<i>Eidolon helvum</i>)	Ghana
HE801056	BatPV/Eidolon/RC0 EPMV_RC09_236s2	Bat (<i>Eidolon helvum</i>)	Republic of the Congo
HQ660153	BatPV/Hipposideros/GH19-140/GHA/2009	Bat (<i>Hipposideros ruber</i>)	Ghana
HQ660155	BatPV/Coleura/GAB/2009	Bat (<i>Coleura afra</i>)	Gabon
HQ660158	BatPV/Hipposideros/GB09790/GAB/2009	Bat (<i>Hipposideros caffer</i>)	Gabon
HQ660159	BatPV/Hipposideros/GB09898/GAB/2009	Bat (<i>Hipposideros gigas</i>)	Gabon
HQ660162	BatPV/Hipposideros/GB59-59/GHA/2009	Bat (<i>Hipposideros abae</i>)	Ghana
HQ660166	BatPV/Myotis/N78-14/GER/2008	Bat (<i>Myotis myotis</i>)	Germany
HQ660170	BatPV/Myotis/NM98-46/GER/2008	Bat (<i>Myotis bechsteinii</i>)	Germany
HQ660195	RodentPV/Rattus/TP180/THA/2007	Black rat (<i>Rattus rattus</i>)	Thailand
JN862578	BatPV/Eidolon/GHA/2010	Bat (<i>Eidolon helvum</i>)	Ghana
JQ411014	Feline morbillivirus	Cat (<i>Felis catus</i>)	Hong Kong
KC538903	BatPV/Hipposideros/NIG955/NIG/2010	Bat (<i>Hipposideros</i> sp.)	Nigeria
KC578581	BatPV/Nycteris/RSA-855/2010	Bat (<i>Nycteris thebaica</i>)	South Africa
KC578675	BatPV/Hipposideros/DRC-328/2011	Bat (<i>Hipposideros fuliginosus</i>)	Democratic Republic of the Congo
KC578678	BatPV/Rhinolophus/CAM-45/2010	Bat (<i>Rhinolophus</i> sp.)	Cameroon
KC578681	BatPV/Hipposideros/CAM-88/2010	Bat (<i>Hipposideros</i> sp.)	Cameroon

GenBank Accession	Virus Identity	Host	Location
KC802221	Phocine distemper virus	Harbour seal (<i>Phoca vitulina</i>)	Netherlands
MF943130	Bank vole virus/RP-12	Bank vole (<i>Myodes glareolus</i>)	Russia: Bashkiria
MK967695	BatPV/Hipposideros/UP450/RWA/2008	Bat (<i>Hipposideros ruber</i>)	Rwanda
MK967696	BatPV/Otomops/UP535/RWA/2008	Bat (<i>Otomops martiensseni</i>)	Rwanda
MT085305	Paramyxoviridae sp. RtRt-ParaV/TI2006	Tanezumi rat (<i>Rattus tanezumi</i>)	Thailand
NC_001498	Measles virus	‡	‡
NC_001552	Sendai virus	Rodents	‡
NC_001906	Hendra virus	‡	‡
NC_001921	Canine distemper virus	‡	‡
NC_002161	Bovine parainfluenza virus 3	Lab Host (Madin Darby bovine kidney cells)	‡
NC_002199	Tupaia paramyxovirus	‡	‡
NC_002200	Mumps virus	‡	‡
NC_002728	Nipah virus	‡	‡
NC_003461	Human parainfluenza virus 1	‡	‡
NC_005339	Mossman virus	Wild rat	‡
NC_007454	J-virus	House mouse (<i>Mus musculus</i>)	Australia
NC_017937	Nariva virus	Wild rodent (<i>Zygodontomys brevicauda</i>)	Trinidad and Tobago
NC_025256	BatPV/Eid_hel/GH-M74a/GHA/2009	Bat (<i>Eidolon helvum</i>)	Ghana
NC_025351	Cedar virus	Bat (<i>Pteropus</i> sp.)	Australia
NC_025352	Mojiang virus	<i>Rattus rattus sladeni</i>	China
NC_025355	Tailam virus	Sikkim rat (<i>Rattus andamanensis</i>)	Hong Kong
NC_025402	Porcine parainfluenza virus 1	Pig (<i>Sus scrofa domesticus</i>)	Hong Kong
NC_043540	Mount Mabu Lophuromys virus 2	Brush-furred mouse (<i>Lophuromys machangui</i>)	Mozambique
OK623360	Denalis virus strain BE/Ninove/Mar/1/2019	Common vole (<i>Microtus arvalis</i>)	Belgium
OK623365	Meleucus virus strain GN/Meliandou/Me/2/2018	Guinea multimammate mouse (<i>Mastomys erythroleucus</i>)	Guinea
X98291	Rinderpest virus	Artiodactyls	Kenya
Z11575	Human parainfluenza virus 3	‡	‡
NC_025386	Salem virus	Horse	USA
DQ172679	Pacific salmon paramyxovirus/RinLyWA99/USA	Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	USA
EU156171	Atlantic salmon paramyxovirus/Yrkje371/95/NOR	‡	Norway
AY141760	Fer-de-lance virus	‡	‡

GenBank Accession	Virus Identity	Host	Location
KX588254	Ferlavirus sp.	Gila monster (<i>Heloderma suspectum</i>)	USA
MH900516	Salmon Aquaparamyxovirus	Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	USA

* Information not listed

Shaded information indicates sequences included in PV analyses-based SCR assay length, unshaded sequences were included in phylogeny based on both RMH and SCR assays.

Table 7.5: Pairwise amino acid sequence identity for RMH RT-PCR sequences

Clustering done by identifying sequences that share at least 94 amino acid identity. Pairwise comparison of a single sequence with all other sequences done to determine if the identity is above the 94.0% threshold. Sequences that fell below the threshold were considered as separate species. For a single cluster, a presentative sequence was used in further analysis. Information on unique sequences and representative sequences and members that share the same cluster are indicated below the table.

Seq->	DON009B008	DON009B017 ¹	DON009B018	DON009B025	DON007B005 [†]	DON007B007 ²	DON007B008	DON007B010	DON007B011	DON007B012	DON007B015	DON007B021	DON007B022	DON007B023	DON007B024	DON007B025	DON007B026	DON007B028	DON007B029	DON007B030	DON007B033	DON007B035	DON007B039	DON007B040	DON007B043	DON007B047	DON010B009 ³	DON010B024
BatPV/Hipposideros/DON009B008/BW/2021	ID	75.3	75.3	75.3	61.5	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	70.2	99.2	75.3
BatPV/Hipposideros/DON009B017/BW/2021 ¹		ID	100.0	100.0	64.4	71.0	71.0	71.0	70.2	70.2	71.0	70.2	70.2	71.0	70.2	71.0	70.2	71.0	71.0	71.0	71.0	71.0	70.2	70.2	70.2	71.0	76.0	100.0
BatPV/Hipposideros/DON009B018/BW/2021			ID	100.0	64.4	71.0	71.0	71.0	70.2	70.2	71.0	70.2	70.2	71.0	70.2	71.0	70.2	71.0	71.0	71.0	71.0	71.0	70.2	70.2	70.2	71.0	76.0	100.0
BatPV/Hipposideros/DON009B025/BW/2021				ID	64.4	71.0	71.0	71.0	70.2	70.2	71.0	70.2	70.2	71.0	70.2	71.0	70.2	71.0	71.0	71.0	71.0	71.0	70.2	70.2	70.2	71.0	76.0	100.0
BatPV/Nycteris/DON007B005/BW/2022 [†]					ID	78.9	78.9	78.9	79.7	78.9	79.7	79.7	79.7	78.9	79.7	78.9	79.7	78.9	78.9	78.9	78.9	78.9	79.7	79.7	79.7	78.9	62.3	64.4
BatPV/Nycteris/DON007B007/BW/2022 ²						ID	100.0	100.0	99.2	100.0	99.2	99.2	99.2	100.0	99.2	100.0	100.0	100.0	99.2	100.0	100.0	100.0	99.2	99.2	99.2	100.0	71.0	71.0
BatPV/Nycteris/DON007B008/BW/2022							ID	100.0	99.2	100.0	99.2	99.2	100.0	100.0	99.2	100.0	100.0	100.0	99.2	100.0	100.0	99.2	99.2	99.2	100.0	71.0	71.0	71.0
BatPV/Nycteris/DON007B010/BW/2022								ID	99.2	100.0	99.2	99.2	100.0	100.0	99.2	100.0	100.0	100.0	99.2	100.0	100.0	99.2	99.2	99.2	100.0	71.0	71.0	71.0
BatPV/Nycteris/DON007B011/BW/2022									ID	100.0	99.2	100.0	100.0	100.0	99.2	100.0	100.0	100.0	98.5	99.2	99.2	100.0	100.0	100.0	99.2	71.0	71.0	70.2
BatPV/Nycteris/DON007B012/BW/2022										ID	99.2	100.0	100.0	100.0	99.2	100.0	100.0	99.2	99.2	98.5	99.2	99.2	100.0	100.0	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B015/BW/2022											ID	99.2	99.2	100.0	99.2	100.0	100.0	100.0	99.2	100.0	100.0	99.2	99.2	99.2	100.0	71.0	71.0	71.0
BatPV/Nycteris/DON007B021/BW/2022												ID	100.0	100.0	99.2	100.0	100.0	99.2	99.2	98.5	99.2	99.2	100.0	100.0	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B022/BW/2022													ID	99.2	100.0	99.2	100.0	99.2	99.2	98.5	99.2	99.2	100.0	100.0	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B023/BW/2022														ID	99.2	100.0	99.2	100.0	99.2	99.2	99.2	99.2	99.2	99.2	100.0	71.0	71.0	71.0
BatPV/Nycteris/DON007B024/BW/2022															ID	99.2	100.0	99.2	99.2	98.5	99.2	99.2	100.0	100.0	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B025/BW/2022																ID	99.2	100.0	100.0	99.2	100.0	100.0	99.2	99.2	100.0	71.0	71.0	71.0

Seq->	DON009B008	DON009B017 ¹	DON009B018	DON009B025	DON007B005 ⁺	DON007B007 ²	DON007B008	DON007B010	DON007B011	DON007B012	DON007B015	DON007B021	DON007B022	DON007B023	DON007B024	DON007B025	DON007B026	DON007B028	DON007B029	DON007B030	DON007B033	DON007B035	DON007B039	DON007B040	DON007B043	DON007B047	DON010B009 ³	DON010B024
BatPV/Nycteris/DON007B026/BW/2022																	ID	99.2	99.2	98.5	99.2	99.2	100.0	100.0	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B028/BW/2022																		ID	100.0	99.2	100.0	100.0	99.2	99.2	99.2	100.0	71.0	71.0
BatPV/Nycteris/DON007B029/BW/2022																			ID	99.2	100.0	100.0	99.2	99.2	99.2	100.0	71.0	71.0
BatPV/Nycteris/DON007B030/BW/2022																				ID	99.2	99.2	98.5	98.5	98.5	99.2	71.0	71.0
BatPV/Nycteris/DON007B033/BW/2022																					ID	100.0	99.2	99.2	99.2	100.0	71.0	71.0
BatPV/Nycteris/DON007B035/BW/2022																						ID	99.2	99.2	99.2	100.0	71.0	71.0
BatPV/Nycteris/DON007B039/BW/2022																							ID	100.0	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B040/BW/2022																								ID	100.0	99.2	71.0	70.2
BatPV/Nycteris/DON007B043/BW/2022																									ID	99.2	71.0	70.2
BatPV/Nycteris/DON007B047/BW/2022																										ID	71.0	71.0
BatPV/Rhinolophus/DON010B009/BW/2021 ³																											ID	76.0
BatPV/Rhinolophus/DON010B024/BW/2021																												ID

Sequences falling within cluster with representative sequence:

¹ BatPV/Hipposideros/DON009B025/BW/2021, BatPV/Hipposideros/DON009B018/BW/2021 and BatPV/Nycteris/DON010B024/BW/2021

² BatPV/Nycteris/DON007B008/BW/2020; BatPV/Nycteris/DON007B010/BW/2020; BatPV/Nycteris/DON007B015/BW/2020; BatPV/Nycteris/DON007B021/BW/2020; BatPV/Nycteris/DON007B022/BW/2020; BatPV/Nycteris/DON007B023/BW/2020; BatPV/Nycteris/DON007B024/BW/2020; BatPV/Nycteris/DON007B025/BW/2020; BatPV/Nycteris/DON007B026/BW/2020; BatPV/Nycteris/DON007B028/BW/2020; BatPV/Nycteris/DON007B029/BW/2020; BatPV/Nycteris/DON007B030/BW/2020; BatPV/Nycteris/DON007B033/BW/2020; BatPV/Nycteris/DON007B035/BW/2020; BatPV/Nycteris/DON007B039/BW/2020; BatPV/Nycteris/DON007B040/BW/2020; BatPV/Nycteris/DON007B043/BW/2020; BatPV/Nycteris/DON007B047/BW/2020

³ BatPV/Hipposideros/DON009B008/BW/2021

* unique sequence

Table 7.6: Uncorrected p- distance for the evolutionary divergence between selected sequences of the partial polymerase (L) gene

The number of base differences per site from between sequences are shown as percentages. Standard error estimate(s) are shown above the diagonal in blue and were obtained by using analytical formulas. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.46). This analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were 410 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

	DON009B017 ¹	DON007B005 [†]	DON007B007 ²	DON010B009 ³	DQ100461	HQ660153	HQ660155	HQ660158	HQ660159	HQ660162	HQ660166	HQ660170	KC538903	KC578581	KC578675	KC578678	KC578681	MK967695	MK967696	NC_007454	NC_025355	NC_043540
BatPV/Hipposideros/DON009B017/BW/2021 ¹		2.3	2.3	2.2	2.3	2.2	2.3	2.3	2.3	2.3	2.3	2.3	2.2	2.3	2.2	2.2	2.2	2.2	2.3	2.3	2.3	2.3
BatPV/Nycteris/DON007B005/BW/2020 [†]	33.2		2.3	2.4	2.4	2.3	2.4	2.4	2.4	2.4	2.3	2.4	2.4	2.2	2.3	2.4	2.3	2.3	2.4	2.4	2.3	2.3
BatPV/Nycteris/DON007B007/BW/2020 ²	32.0	29.5		2.3	2.3	2.2	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.1	2.2	2.3	2.2	2.2	2.3	2.3	2.3	2.3
BatPV/Rhinolophus/DON010B009/BW/2021 ³	26.3	34.9	34.1		2.3	2.3	2.3	2.4	2.2	2.3	2.3	2.3	2.2	2.3	2.3	2.3	2.2	2.3	2.3	2.4	2.3	2.3
DQ100461_Beilon virus	34.1	37.8	33.7	32.7		2.3	2.4	2.4	2.4	2.4	2.3	2.4	2.4	2.3	2.3	2.4	2.3	2.3	2.3	2.2	2.1	2.3
HQ660153_BatPV/Hipposideros/GH19-140/GHA/2009	29.0	34.1	27.8	30.5	33.7		2.3	2.2	2.2	2.2	2.2	2.3	2.1	2.2	1.9	2.2	2.1	2.1	2.2	2.3	2.3	2.3
HQ660155.1_BatPV/Coleura/GAB/2009	31.7	35.9	32.7	33.9	34.9	30.7		2.4	2.3	1.9	2.3	2.3	2.2	2.2	2.3	2.3	2.3	2.3	2.3	2.4	2.3	2.4
HQ660158_BatPV/Hipposideros/GB09790/GAB/2009	30.7	35.9	32.9	35.6	34.9	28.3	35.4		2.3	2.3	2.3	2.2	2.2	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
HQ660159_BatPV/_Hipposideros/GB09898/GAB/2009	30.0	36.6	31.7	28.5	34.9	28.5	31.2	33.2		2.3	2.3	2.3	2.2	2.3	2.2	2.2	2.3	2.3	2.2	2.3	2.3	2.3
HQ660162_BatPV/Hipposideros/GB59-59/GHA/2009	31.2	36.1	31.2	33.9	35.9	29.0	18.3	33.9	30.2		2.3	2.3	2.2	2.3	2.2	2.3	2.3	2.3	2.3	2.4	2.4	2.4
HQ660166_BatPV/_Myotis/N78-14/GER/2008	30.7	33.4	31.0	33.9	34.4	28.0	31.0	30.5	30.7	29.8		2.1	2.2	2.3	2.4	2.3	2.3	2.3	2.3	2.3	2.3	2.4
HQ660170_BatPV/_Myotis/NM98-46/GER/2008	32.9	37.6	33.7	33.2	35.6	31.2	33.9	29.3	33.7	32.4	23.4		2.3	2.3	2.4	2.3	2.3	2.3	2.4	2.4	2.4	2.4
KC538903_BatPV/NIG955/NIG/2010	27.8	35.1	29.8	28.3	35.6	23.7	27.8	29.3	26.6	28.5	29.0	31.0		2.3	2.1	2.2	1.9	2.0	2.2	2.4	2.3	2.3
KC578581_BatPV/Nycteris/RSA-855/2010	30.2	27.1	23.4	31.7	31.7	29.0	29.3	32.0	32.7	31.0	31.5	32.2	30.0		2.2	2.3	2.3	2.2	2.3	2.4	2.3	2.3
KC578675_BatPV/Hipposideros/DRC-328/2011	28.8	33.9	29.0	30.7	32.2	17.1	32.0	30.5	29.0	29.0	34.9	34.9	23.9	29.0		2.2	2.1	2.1	2.2	2.3	2.3	2.3
KC578678_BatPV/Rhinolophus/CAM-45/2010	29.0	36.3	31.0	30.2	36.3	26.6	33.7	34.6	28.5	31.5	33.9	33.2	26.6	32.9	25.9		2.2	2.2	2.3	2.3	2.3	2.3
KC578681_BatPV/Hipposideros/CAM-88/2010	27.3	32.2	29.3	28.8	34.1	25.4	31.2	32.2	30.2	31.5	31.0	32.9	19.0	30.2	24.9	26.6		2.0	2.2	2.4	2.3	2.3
MK967695_BatPV/Hipposideros/UP450/RWA/2008	26.8	32.9	29.3	31.2	32.4	23.7	29.5	30.2	29.5	33.4	30.7	31.5	19.5	29.0	25.4	27.8	21.7		2.2	2.3	2.3	2.3
MK967696_BatPV/Otomops/UP535/RWA/2008	31.7	34.9	31.5	30.0	30.5	26.1	32.7	33.7	28.3	29.8	32.4	34.9	25.9	30.7	26.8	31.5	27.3	26.8		2.3	2.3	2.3
NC_007454_J-virus	31.5	36.3	34.4	34.9	29.3	32.7	35.9	34.1	32.4	38.3	33.4	35.1	36.6	35.6	34.1	34.6	34.9	33.9	33.7		2.2	2.2
NC_025355_Tailam virus	30.7	33.2	30.0	31.2	25.4	34.4	34.4	33.2	34.4	37.3	33.4	37.3	32.9	31.0	34.1	34.1	30.0	32.0	32.4	28.5		2.2
NC_043540_Mount_Mabu_Lophuromys_virus_2	31.7	33.9	31.7	33.9	33.4	32.9	36.6	34.1	33.4	35.6	35.4	35.6	33.9	32.2	32.7	34.1	31.7	32.9	33.7	28.8	28.3	

Sequences represented:

¹ BatPV/Hipposideros/DON009B025/BW/2021, BatPV/Hipposideros/DON009B018/BW/2021 and BatPV/Nycteris/DON010B024/BW/2021

² BatPV/Nycteris/DON007B008/BW/2020; BatPV/Nycteris/DON007B010/BW/2020; BatPV/Nycteris/DON007B015/BW/2020; BatPV/Nycteris/DON007B021/BW/2020;

BatPV/Nycteris/DON007B022/BW/2020; BatPV/Nycteris/DON007B023/BW/2020; BatPV/Nycteris/DON007B024/BW/2020; BatPV/Nycteris/DON007B025/BW/2020; BatPV/Nycteris/DON007B026/BW/2020;

BatPV/Nycteris/DON007B028/BW/2020; BatPV/Nycteris/DON007B029/BW/2020; BatPV/Nycteris/DON007B030/BW/2020; BatPV/Nycteris/DON007B033/BW/2020; BatPV/Nycteris/DON007B035/BW/2020; BatPV/Nycteris/DON007B039/BW/2020; BatPV/Nycteris/DON007B040/BW/2020; BatPV/Nycteris/DON007B043/BW/2020; BatPV/Nycteris/DON007B047/BW/2020
³ BatPV/Hipposideros/DON009B008/BW/2021
 * unique sequence

Table 7.7: Pairwise amino acid sequence identity for SCR RT-PCR derived sequences

Clustering done by identifying sequences that share at least 94.0% amino acid identity. Pairwise comparison of a single sequence with all other sequences done to determine if the identity is above the 94.0% threshold. Sequences that fell below the threshold were considered as separate species. For a single cluster, a presentative sequence was used in further analysis. Information on unique sequences and representative sequences and members that share the same cluster are indicated below the table

Seq->	DON009B005 ¹	DON007B009	DON007B009	DON007B018_A	DON007B018_B ⁺	DON007B019 ²	DON007B020	DON007B027	DON007B031_A	DON007B031_B	DON007B034	DON007B036	DON007B037_A	DON007B037_B	DON007B038	DON007B042_A ⁺	DON007B042_B ⁺	DON007B044	DON007B048_A	DON007B048_B	DON007B049	DON007B051	DON006B030	DON010B023
BatPV/Hipposideros/DON009B005/BW/2021 ¹	ID	63.5	64.8	63.5	63.5	64.8	64.8	63.5	64.8	64.8	64.8	63.5	64.8	64.8	64.8	60.8	62.1	64.8	64.8	63.5	64.8	63.5	60.8	100.0
BatPV/Nycteris/DON007B009/BW/2021_A		ID	93.2	98.6	86.4	98.6	98.6	95.9	91.8	98.6	98.6	90.5	97.2	91.8	98.6	86.4	86.4	98.6	91.8	94.5	98.6	100.0	62.1	63.5
BatPV/Nycteris/DON007B009/BW/2021_B			ID	93.2	91.8	94.5	94.5	93.2	98.6	94.5	94.5	97.2	93.2	98.6	94.5	91.8	90.5	94.5	98.6	90.5	94.5	93.2	62.1	64.8
BatPV/Nycteris/DON007B018/BW/2021_A				ID	86.4	98.6	98.6	95.9	91.8	98.6	98.6	90.5	97.2	91.8	98.6	86.4	87.8	98.6	91.8	94.5	98.6	98.6	62.1	63.5
BatPV/Nycteris/DON007B018/BW/2021_B ⁺					ID	87.8	87.8	86.4	90.5	87.8	87.8	89.1	86.4	90.5	87.8	85.1	98.6	87.8	90.5	83.7	87.8	86.4	63.5	63.5
BatPV/Nycteris/DON007B019/BW/2021 ²						ID	100.0	97.2	93.2	100.0	100.0	91.8	98.6	93.2	100.0	87.8	86.4	100.0	93.2	95.9	100.0	98.6	63.5	64.8
BatPV/Nycteris/DON007B020/BW/2021_							ID	97.2	93.2	100.0	100.0	91.8	98.6	93.2	100.0	87.8	86.4	100.0	93.2	95.9	100.0	98.6	63.5	64.8
BatPV/Nycteris/DON007B027/BW/2021								ID	91.8	97.2	97.2	90.5	95.9	91.8	97.2	86.4	85.1	97.2	91.8	95.9	97.2	95.9	62.1	63.5
BatPV/Nycteris/DON007B031/BW/2021_A									ID	93.2	93.2	98.6	93.2	100.0	93.2	93.2	89.1	93.2	100.0	89.1	93.2	91.8	62.1	64.8
BatPV/Nycteris/DON007B031/BW/2021_B										ID	100.0	91.8	98.6	93.2	100.0	87.8	86.4	100.0	93.2	95.9	100.0	98.6	63.5	64.8
BatPV/Nycteris/DON007B034/BW/2021											ID	91.8	98.6	93.2	100.0	87.8	86.4	100.0	93.2	95.9	100.0	98.6	63.5	64.8
BatPV/Nycteris/DON007B036/BW/2021_												ID	91.8	98.6	91.8	91.8	87.8	91.8	98.6	87.8	91.8	90.5	60.8	63.5
BatPV/Nycteris/DON007B037/BW/2021_A													ID	93.2	98.6	87.8	85.1	98.6	93.2	94.5	98.6	97.2	63.5	64.8
BatPV/Nycteris/DON007B037/BW/2021_B														ID	93.2	93.2	89.1	93.2	100.0	89.1	93.2	91.8	62.1	64.8
BatPV/Nycteris/DON007B038/BW/2021															ID	87.8	86.4	100.0	93.2	95.9	100.0	98.6	63.5	64.8
BatPV/Nycteris/DON007B042/BW/2021_A																ID	83.7	87.8	93.2	83.7	87.8	86.4	60.8	60.8
BatPV/Nycteris/DON007B042/BW/2021_B																	ID	86.4	89.1	82.4	86.4	86.4	62.1	62.1
BatPV/Nycteris/DON007B044/BW/2021																		ID	93.2	95.9	100.0	98.6	63.5	64.8
BatPV/Nycteris/DON007B048/BW/2021_A ⁺																			ID	89.1	93.2	91.8	62.1	64.8
BatPV/Nycteris/DON007B048/BW/2021_B ⁺																				ID	95.9	94.5	59.4	63.5
BatPV/Nycteris/DON007B049/BW/2021																					ID	98.6	63.5	64.8

[illegible]

Table 7.8: Uncorrected p- distance for the evolutionary divergence between selected sequences of the partial polymerase (L) gene

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1		3.7	3.6	3.7	3.7	3.6	3.7	3.7	3.6	3.6	3.6	3.6	3.6	3.7	3.6	3.7	3.7	3.7	3.7	3.7	3.7	3.8	3.6	3.7	3.7	3.9	3.7	3.7	3.7	3.6	3.7	3.7	3.8	3.7	3.7	3.8
2	34.1		3.1	1.0	3.3	1.0	1.0	1.0	3.1	3.1	1.0	3.1	3.2	3.3	3.1	1.4	1.2	3.7	3.6	3.6	3.6	3.7	3.7	3.6	3.8	3.9	3.7	3.0	3.6	3.7	3.5	3.5	3.7	3.7	3.6	3.7
3	31.7	19.8		3.1	3.1	3.0	3.1	3.1	0.8	0.8	3.1	0.8	1.6	3.1	0.8	3.1	3.0	3.7	3.7	3.6	3.7	3.7	3.6	3.6	3.8	3.9	3.7	3.2	3.6	3.6	3.6	3.6	3.7	3.7	3.6	3.8
4	33.5	1.8	19.8		3.2	0.6	0.8	1.0	3.1	3.1	0.8	3.1	3.2	3.2	3.1	1.2	1.2	3.7	3.6	3.6	3.6	3.7	3.7	3.7	3.8	3.9	3.7	3.1	3.6	3.7	3.5	3.5	3.7	3.7	3.6	3.7
5	33.5	24.0	21.0	22.8		3.2	3.3	3.2	3.1	3.2	3.3	3.1	3.3	0.6	3.1	3.2	3.3	3.8	3.8	3.7	3.7	3.8	3.7	3.7	3.8	3.9	3.7	3.4	3.7	3.8	3.6	3.7	3.7	3.7	3.6	3.8
6	32.9	1.8	19.2	0.6	22.2		0.8	0.8	3.0	3.1	1.0	3.0	3.1	3.2	3.0	1.0	1.0	3.7	3.6	3.6	3.6	3.7	3.7	3.7	3.8	3.9	3.7	3.1	3.6	3.7	3.5	3.5	3.7	3.7	3.6	3.7
7	34.1	1.8	20.4	1.2	23.4	1.2		1.2	3.1	3.2	1.2	3.1	3.2	3.2	3.1	1.3	1.3	3.7	3.6	3.6	3.6	3.7	3.7	3.6	3.8	3.9	3.6	3.0	3.6	3.6	3.5	3.5	3.7	3.7	3.6	3.7
8	33.5	1.8	20.4	1.8	22.8	1.2	2.4		3.1	3.2	1.0	3.1	3.2	3.2	3.1	1.3	1.3	3.7	3.6	3.6	3.6	3.7	3.7	3.7	3.8	3.9	3.7	3.0	3.6	3.6	3.5	3.5	3.7	3.7	3.5	3.7
9	30.5	21.0	1.2	19.8	21.0	19.2	20.4	20.4		0.8	3.1	0.0	1.3	3.1	0.0	3.1	3.1	3.7	3.7	3.5	3.7	3.7	3.6	3.6	3.7	3.9	3.7	3.2	3.6	3.6	3.6	3.6	3.7	3.7	3.6	3.8
10	31.7	21.0	1.2	21.0	22.2	20.4	21.6	21.6	1.2		3.2	0.8	1.6	3.2	0.8	3.1	3.1	3.7	3.7	3.6	3.7	3.7	3.7	3.7	3.8	3.9	3.7	3.2	3.6	3.6	3.6	3.6	3.7	3.7	3.6	3.8
11	32.9	1.8	21.0	1.2	24.0	1.8	2.4	1.8	21.0	22.2		3.1	3.2	3.3	3.1	1.4	1.4	3.7	3.6	3.6	3.6	3.7	3.7	3.7	3.8	3.9	3.7	3.0	3.6	3.7	3.5	3.6	3.7	3.7	3.6	3.7
12	30.5	21.0	1.2	19.8	21.0	19.2	20.4	20.4	0.0	1.2	21.0		1.3	3.1	0.0	3.1	3.1	3.7	3.7	3.5	3.7	3.7	3.6	3.6	3.7	3.9	3.7	3.2	3.6	3.6	3.6	3.6	3.7	3.7	3.6	3.8
13	32.9	22.8	4.2	21.6	24.0	21.0	22.2	22.2	3.0	4.2	22.8	3.0		3.3	1.3	3.2	3.2	3.8	3.7	3.6	3.7	3.7	3.7	3.7	3.8	3.9	3.7	3.4	3.6	3.6	3.6	3.6	3.7	3.7	3.7	3.8
14	33.5	23.4	21.0	22.2	0.6	21.6	22.8	22.2	21.0	22.2	23.4	21.0	24.0		3.1	3.2	3.2	3.8	3.7	3.7	3.7	3.8	3.7	3.7	3.8	3.9	3.7	3.4	3.7	3.8	3.6	3.6	3.7	3.7	3.6	3.8
15	30.5	21.0	1.2	19.8	21.0	19.2	20.4	20.4	0.0	1.2	21.0	0.0	3.0	21.0		3.1	3.1	3.7	3.7	3.5	3.7	3.7	3.6	3.6	3.7	3.9	3.7	3.2	3.6	3.6	3.6	3.6	3.7	3.6	3.8	
16	33.5	3.6	19.8	2.4	22.8	1.8	3.0	3.0	19.8	21.0	3.6	19.8	22.2	22.2	19.8		1.4	3.7	3.7	3.6	3.6	3.7	3.7	3.7	3.8	3.9	3.7	3.1	3.6	3.7	3.5	3.5	3.7	3.7	3.6	3.8
17	34.1	2.4	19.2	2.4	23.4	1.8	3.0	3.0	20.4	20.4	3.6	20.4	22.2	22.8	20.4	3.6		3.7	3.7	3.6	3.7	3.7	3.7	3.7	3.8	3.9	3.7	3.1	3.7	3.7	3.6	3.5	3.7	3.7	3.6	3.8
18	35.3	35.3	37.1	35.3	38.9	34.7	35.9	33.5	35.9	37.1	35.3	35.9	37.7	38.3	35.9	35.9			2.1	3.8	3.8	3.8	3.8	3.8	3.8	3.9	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.7	3.5	3.8

19	34. 1	32. 3	34. 7	32. 3	37. 7	32. 9	32. 3	32. 3	34. 1	34. 7	32. 3	34. 1	35. 9	37. 1	34. 1	34. 1	33. 5	8.4			3.8	3.8	3.8	3.8	3.8	3.8	3.9	3.8	3.7	3.7	3.8	3.8	3.7	3.7	3.5	3.3	3.7	
20	35. 3	32. 3	30. 5	31. 7	34. 7	31. 1	31. 1	31. 7	29. 3	30. 5	32. 3	29. 3	31. 7	34. 1	29. 3	31. 1	32. 9	41. 3	37. 7			3.6	3.6	3.6	3.6	3.7	3.9	3.3	3.7	2.9	3.5	3.4	3.3	3.6	3.8	3.8	3.7	
21	36. 5	32. 3	33. 5	32. 3	37. 1	32. 3	31. 7	32. 9	33. 5	34. 1	32. 3	33. 5	35. 3	36. 5	33. 5	32. 3	33. 5	42. 5	38. 9	30. 5			3.8	3.7	2.8	3.8	3.9	3.5	3.5	3.5	3.8	3.6	3.6	3.7	3.8	3.8	3.8	
22	38. 3	34. 1	35. 3	34. 1	38. 3	33. 5	34. 7	34. 1	34. 7	35. 3	34. 7	34. 7	36. 5	37. 7	34. 7	35. 3	34. 1	41. 9	39. 5	31. 7	37. 7			3.7	3.8	3.6	3.9	3.6	3.7	3.7	3.8	3.6	3.7	3.8	3.7	3.8	3.8	
23	30. 5	34. 7	32. 9	34. 7	33. 5	34. 1	34. 7	32. 9	33. 5	34. 7	32. 9	34. 7	34. 7	33. 5	32. 9	35. 3	35. 3	43. 7	41. 3	30. 5	34. 1	34. 1			3.7	3.8	3.9	3.5	3.7	3.6	3.7	3.5	3.6	3.6	3.7	3.8	3.7	
24	37. 1	32. 9	32. 9	33. 5	36. 5	33. 5	32. 3	33. 5	32. 9	33. 5	33. 5	32. 9	34. 7	36. 5	32. 9	34. 1	34. 7	43. 1	38. 9	30. 5	15. 0	37. 7	33. 5			3.8	3.9	3.5	3.6	3.5	3.7	3.5	3.6	3.6	3.8	3.8	3.7	
25	34. 1	39. 5	37. 7	39. 5	40. 1	38. 9	38. 9	38. 9	37. 1	37. 7	40. 1	37. 1	38. 9	39. 5	37. 1	40. 1	39. 5	41. 3	38. 3	35. 9	40. 1	30. 5	38. 3	41. 3			3.9	3.7	3.8	3.8	3.7	3.7	3.7	3.8	3.8	3.8	3.8	
26	46. 7	47. 3	46. 7	47. 9	52. 1	47. 9	46. 5	48. 5	46. 7	46. 7	49. 1	46. 7	46. 7	52. 7	46. 7	49. 1	48. 5	52. 1	47. 9	50. 9	52. 7	51. 5	49. 1	49. 1			3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	
27	34. 1	33. 5	34. 1	34. 1	34. 7	34. 1	32. 9	34. 1	34. 1	34. 7	34. 1	34. 1	35. 9	34. 7	34. 1	34. 1	34. 7	44. 3	40. 1	23. 4	28. 7	31. 7	28. 1	28. 1	36. 5	49. 1			3.6	3.3	3.5	2.8	3.0	3.6	3.8	3.7	3.8	
28	36. 5	18. 2	22. 8	19. 8	26. 3	19. 8	18. 6	19. 2	22. 2	22. 8	19. 2	22. 2	25. 1	25. 7	22. 2	20. 4	21. 0	38. 3	33. 5	34. 1	28. 1	37. 1	36. 5	32. 9	38. 9	46. 7	31. 7			3.7	3.7	3.5	3.6	3.7	3.8	3.6	3.7	
29	35. 9	31. 7	31. 1	32. 9	37. 1	32. 9	31. 7	32. 9	30. 5	31. 1	32. 9	30. 5	32. 9	37. 1	30. 5	32. 9	34. 1	39. 5	35. 3	17. 4	29. 9	33. 5	32. 3	27. 5	39. 5	50. 9	23. 4	33. 5			3.4	3.2	3.4	3.6	3.7	3.7	3.7	
30	31. 1	33. 5	31. 7	34. 1	38. 9	34. 1	32. 9	32. 9	30. 5	31. 7	34. 1	30. 5	31. 7	38. 9	30. 5	34. 1	35. 3	41. 3	38. 9	27. 5	38. 9	40. 7	34. 7	36. 5	36. 5	47. 3	28. 7	34. 7	26. 9			3.3	3.5	3.8	3.8	3.8	3.7	
31	34. 1	29. 3	30. 5	29. 9	32. 9	29. 9	28. 7	29. 9	30. 5	31. 1	29. 9	30. 5	32. 9	32. 9	30. 5	29. 9	30. 5	41. 9	37. 7	25. 1	30. 5	32. 3	29. 3	29. 3	34. 7	49. 1	15. 6	28. 7	22. 8	24. 6			3.0	3.5	3.7	3.7	3.7	3.7
32	35. 3	29. 9	31. 7	29. 9	33. 5	29. 3	29. 3	29. 9	31. 1	31. 7	30. 5	31. 7	32. 9	32. 9	31. 1	29. 3	29. 3	40. 1	37. 1	24. 6	31. 1	34. 1	31. 1	32. 9	34. 1	51. 5	19. 2	30. 5	26. 3	28. 1	18. 6			3.7	3.7	3.7	3.8	
33	38. 3	35. 3	35. 3	34. 7	33. 5	34. 7	33. 5	35. 3	34. 7	35. 9	35. 3	34. 7	36. 5	33. 5	34. 7	35. 9	36. 5	37. 3	35. 3	30. 5	36. 5	40. 7	32. 9	31. 7	40. 1	49. 7	31. 7	36. 5	31. 7	28. 1	33. 5			3.8	3.8	3.8	3.8	
34	33. 5	33. 5	35. 9	34. 1	36. 5	34. 7	34. 1	34. 7	35. 9	35. 9	34. 1	35. 9	36. 5	36. 5	35. 9	35. 9	34. 7	34. 1	29. 3	38. 3	41. 3	35. 9	33. 5	41. 9	38. 3	47. 3	40. 7	37. 7	40. 7	36. 5	36. 5	38. 3			3.5	3.5	3.5	
35	34. 1	30. 5	32. 9	31. 1	30. 5	30. 5	31. 7	29. 9	32. 9	32. 9	31. 1	32. 3	34. 1	30. 5	32. 3	31. 1	31. 1	27. 5	24. 0	37. 7	40. 7	37. 7	38. 3	39. 5	39. 5	47. 9	35. 9	32. 3	35. 9	39. 1	34. 1	34. 1	37. 7	29. 3		3.7	3.7	3.7
36	38. 9	35. 9	40. 1	37. 1	38. 9	37. 1	37. 1	37. 1	39. 5	40. 1	37. 1	39. 5	40. 1	38. 3	39. 5	37. 7	38. 3	40. 1	35. 9	36. 5	40. 1	38. 3	36. 5	40. 1	39. 5	47. 3	40. 7	36. 5	35. 9	34. 7	37. 1	40. 7	29. 9	33. 5		3.7	3.7	3.7

List of sequences as they appear in the table above: **(1)** BatPV/Hipposideros/DON009B005/BW/2021, **(2)** BatPV/Nycteris/DON007B009/BW/2021_A, **(3)** BatPV/Nycteris/DON007B009/BW/2021_B, **(4)**

BatPV/Nycteris/DON007B018/BW/2021_A, **(5)** BatPV/Nycteris/DON007B018/BW/2021_B, **(6)** BatPV/Nycteris/DON007B019/BW/2021, **(7)** BatPV/Nycteris/DON007B022/BW/2021, **(8)**

BatPV/Nycteris/DON007B027/BW/2021 **(9)** BatPV/Nycteris/DON007B031/BW/2021_A, **(10)** BatPV/Nycteris/DON007B036/BW/2021, **(11)** BatPV/Nycteris/DON007B037/BW/2021_A, **(12)**

BatPV/Nycteris/DON007B037/BW/2021_B, **(13)** BatPV/Nycteris/DON007B042/BW/2021_A, **(14)** BatPV/Nycteris/DON007B042/BW/2021_B, **(15)** BatPV/Nycteris/DON007B048/BW/2021_A, **(16)**

BatPV/Nycteris/DON007B048/BW/2021_B, **(17)** BatPV/Nycteris/DON007B051/BW/2021, **(18)** BatPV/Rhinolophus/DON006B030/BW/2020, **(19)** DQ100461 Beilong virus, **(20)** HQ660153_BatPV/Hipposideros/GH19-

140/GHA/2009paramyxovirus, **(21)** HQ660155 BatPV/Coleura/GAB/2009, **(22)** HQ660158 BatPV/Hipposideros/GB09790/GAB/2009, **(23)** HQ660159_BatPV/Hipposideros/GB09898/GAB/2009, **(24)** HQ660162

BatPV/Hipposideros/GB59-59/GHA/2009, **(25)** HQ660170 BatPV/Myotis/NM98-46/GER/2008, **(26)** HQ660195 RodentPV/Rattus/TP180/THA/2007, **(27)** KC538903 BatPV/NIG955/NIG/2010, **(28)** KC578581

BatPV/Nycteris/RSA-855/2010, **(29)** KC578675 BatPV/Hipposideros/DRC-328/2011, **(30)** KC578678 BatPV/Rhinolophus/CAM-45/2010, **(31)** KC578681 BatPV/Hipposideros/CAM-88/2010, **(32)**

MK967695_BatPV/Hipposideros/UP450/RWA/2008, **(33)** MK967696_BatPV/Otomops/UP535/RWA/2008, **(34)** NC_007454 J-virus, **(35)** NC_025355 Tailam virus, **(36)** NC_043540 Mount Mabu Lophuromys virus 2

Table 7.9: Pairwise sequence identity of RMH and SCR derived sequences

Sequences obtained using the SCR assay are highlighted in blue, while those from the RMH assay are indicated in red. Identity scores ≥ 90 are highlighted in bold. Nucleotide identities are shown in the upper diagonal (cells are grey), while amino acid identities are shown in the lower diagonal.

Seq->	DON009B005 ¹	DON007B009_A	DON007B009_B	DON007B018_A	DON007B018_B ⁺	DON007B019 ²	DON007B027	DON007B031_A	DON007B036	DON007B037_A	DON007B037_B	DON007B042_A ⁺	DON007B042_B ⁺	DON007B048_A	DON007B048_B	DON007B051	DON006B030 ⁺	DON009B017 ³	DON007B005 ⁺	DON007B007 ⁴	DON010B009 ⁵
BatPV/Hipposideros/DON009B005/BW/2021 ¹	ID	65.8	68.2	66.4	66.4	67.0	66.4	69.4	68.2	67.0	69.4	67.0	66.4	69.4	66.4	65.8	64.6	69.4	63.4	65.8	95.8
BatPV/Nycteris/DON007B009/BW/2021_A	60.0	ID	80.2	98.2	76.0	98.2	98.2	79.0	79.0	98.2	79.0	77.2	76.6	79.0	96.4	97.6	64.6	68.2	73.6	98.8	64.6
BatPV/Nycteris/DON007B009/BW/2021_B	60.0	92.7	ID	80.2	79.0	80.8	79.6	98.8	98.8	79.0	98.8	95.8	79.0	98.8	80.2	80.8	62.8	68.8	77.8	79.0	65.8
BatPV/Nycteris/DON007B018/BW/2021_A	60.0	100.0	92.7	ID	77.2	99.4	98.2	80.2	79.0	98.8	80.2	78.4	77.8	80.2	97.6	97.6	64.6	67.6	74.2	98.2	64.0
BatPV/Nycteris/DON007B018/BW/2021_B ⁺	58.1	83.6	89.0	83.6	ID	77.8	77.2	79.0	77.8	76.0	79.0	76.0	99.4	79.0	77.2	76.6	61.0	67.6	91.6	76.0	63.4
BatPV/Nycteris/DON007B019/BW/2021 ²	60.0	100.0	92.7	100.0	83.6	ID	98.8	80.8	79.6	98.2	80.8	79.0	78.4	80.8	98.2	98.2	65.2	67.6	74.8	98.2	64.0
BatPV/Nycteris/DON007B027/BW/2021	60.0	98.1	92.7	98.1	83.6	98.1	ID	79.6	78.4	98.2	79.6	77.8	77.8	79.6	97.0	97.0	66.4	67.6	73.6	98.2	63.4
BatPV/Nycteris/DON007B031/BW/2021_A	60.0	90.9	98.1	90.9	87.2	90.9	90.9	ID	98.8	79.0	100.0	97.0	79.0	100.0	80.2	79.6	64.0	69.4	77.2	79.0	66.4
BatPV/Nycteris/DON007B036/BW/2021_	58.1	89.0	96.3	89.0	85.4	89.0	89.0	98.1	ID	77.8	98.8	95.8	77.8	98.8	79.0	79.6	62.8	68.8	76.6	77.8	65.8
BatPV/Nycteris/DON007B037/BW/2021_A	60.0	98.1	90.9	98.1	81.8	98.1	96.3	90.9	89.0	ID	79.0	77.2	76.6	79.0	96.4	96.4	64.6	67.6	73.0	98.2	64.6
BatPV/Nycteris/DON007B037/BW/2021_B	60.0	90.9	98.1	90.9	87.2	90.9	90.9	100.0	98.1	90.9	ID	97.0	79.0	100.0	80.2	79.6	64.0	69.4	77.2	79.0	66.4
BatPV/Nycteris/DON007B042/BW/2021_A ⁺	56.3	85.4	90.9	85.4	81.8	85.4	85.4	92.7	90.9	85.4	92.7	ID	76.0	97.0	77.8	77.8	62.2	67.0	74.8	77.2	64.0
BatPV/Nycteris/DON007B042/BW/2021_B ⁺	58.1	83.6	89.0	83.6	100.0	83.6	83.6	87.2	85.4	81.8	87.2	81.8	ID	79.0	77.8	77.2	61.6	68.2	91.0	76.6	63.4
BatPV/Nycteris/DON007B048/BW/2021_A	60.0	90.9	98.1	90.9	87.2	90.9	90.9	100.0	98.1	90.9	100.0	92.7	87.2	ID	80.2	79.6	64.0	69.4	77.2	79.0	66.4
BatPV/Nycteris/DON007B048/BW/2021_B	60.0	96.3	89.0	96.3	80.0	96.3	94.5	87.2	85.4	94.5	87.2	81.8	80.0	87.2	ID	96.4	64.0	67.0	74.2	96.4	63.4
BatPV/Nycteris/DON007B051/BW/2021	60.0	100.0	92.7	100.0	83.6	100.0	98.1	90.9	89.0	98.1	90.9	85.4	83.6	90.9	96.3	ID	64.0	67.6	74.2	96.4	63.4
BatPV/Rhinolophus/DON006B030/BW/2020 ⁺	58.1	61.8	60.0	61.8	61.8	61.8	61.8	60.0	58.1	61.8	60.0	58.1	61.8	60.0	58.1	61.8	ID	59.8	57.4	64.6	62.8
BatPV/Hipposideros/DON009B017/BW/2021 ³	69.0	67.2	67.2	67.2	67.2	67.2	67.2	69.0	67.2	67.2	69.0	63.6	67.2	69.0	67.2	67.2	61.8	ID	66.4	68.8	70.6
BatPV/Nycteris/DON007B005/BW/2020 ⁺	58.1	81.8	87.2	81.8	87.2	81.8	81.8	85.4	83.6	80.0	85.4	80.0	87.2	85.4	78.1	81.8	58.1	67.2	ID	73.0	62.8
BatPV/Nycteris/DON007B007/BW/2020 ⁴	60.0	100.0	92.7	100.0	83.6	100.0	98.1	90.9	89.0	98.1	90.9	85.4	83.6	90.9	96.3	100.0	61.8	67.2	81.8	ID	64.0
BatPV/Rhinolophus/DON010B009/BW/2021 ⁵	96.3	60.0	60.0	60.0	58.1	60.0	60.0	60.0	58.1	60.0	60.0	54.5	58.1	60.0	60.0	60.0	58.1	72.7	58.1	60.0	ID

Sequences within the same cluster:

¹ BatPV/Rhinolophus/DON010B023/BW/2021

² BatPV/Nycteris/DON007B020/BW/2021, BatPV/Nycteris/DON007B031/BW/2021_B, BatPV/Nycteris/DON007B034/BW/2021, BatPV/Nycteris/DON007B038/BW/2021, BatPV/Nycteris/DON007B044/BW/2021 and BatPV/Nycteris/DON007B049/BW/2021

³ BatPV/Hipposideros/DON009B025/BW/2021, BatPV/Hipposideros/DON009B018/BW/2021 and BatPV/Nycteris/DON010B024/BW/2021

⁴ BatPV/Nycteris/DON007B008/BW/2020; BatPV/Nycteris/DON007B010/BW/2020; BatPV/Nycteris/DON007B015/BW/2020; BatPV/Nycteris/DON007B021/BW/2020; BatPV/Nycteris/DON007B022/BW/2020; BatPV/Nycteris/DON007B023/BW/2020; BatPV/Nycteris/DON007B024/BW/2020; BatPV/Nycteris/DON007B025/BW/2020; BatPV/Nycteris/DON007B026/BW/2020; BatPV/Nycteris/DON007B028/BW/2020;

BatPV/Nycteris/DON007B029/BW/2020; BatPV/Nycteris/DON007B030/BW/2020; BatPV/Nycteris/DON007B033/BW/2020; BatPV/Nycteris/DON007B035/BW/2020; BatPV/Nycteris/DON007B039/BW/2020; BatPV/Nycteris/DON007B040/BW/2020; BatPV/Nycteris/DON007B043/BW/2020; BatPV/Nycteris/DON007B047/BW/2020
⁵ BatPV/Hipposideros/DON009B008/BW/2021
 * unique sequence

Table 7.10: Uncorrected p- distance for the evolutionary divergence between detected RMH and SCR sequences of the partial polymerase (L) gene

The number of base differences per site from between sequences are shown as percentages. Standard error estimate(s) are shown above the diagonal and were obtained by using analytical formulas. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.96). This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 167 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Seq ->	DON009B005 ¹	DON007B009_A	DON007B009_B	DON007B018_A	DON007B018_B ⁺	DON007B019 ²	DON007B027	DON007B031_A	DON007B036	DON007B037_A	DON007B037_B	DON007B042_A ⁺	DON007B042_B ⁺	DON007B048_A	DON007B048_B	DON007B051	DON006B030 [†]	DON009B017 ³	DON007B005 [†]	DON007B007 ⁴	DON010B009 ⁵
BatPV/Hipposideros/DON009B005/BW/2021 ¹		3.7	3.6	3.7	3.7	3.6	3.7	3.6	3.6	3.6	3.6	3.6	3.7	3.6	3.7	3.7	3.7	3.6	3.7	3.7	1.6
BatPV/Nycteris/DON007B009/BW/2021_A	34.1		3.1	1.0	3.3	1.0	1.0	3.1	3.1	1.0	3.1	3.2	3.3	3.1	1.4	1.2	3.7	3.6	3.4	0.8	3.7
BatPV/Nycteris/DON007B009/BW/2021_B	31.7	19.8		3.1	3.1	3.0	3.1	0.8	0.8	3.1	0.8	1.6	3.1	0.8	3.1	3.0	3.7	3.6	3.2	3.1	3.7
BatPV/Nycteris/DON007B018/BW/2021_A	33.5	1.8	19.8		3.2	0.6	1.0	3.1	3.1	0.8	3.1	3.2	3.2	3.1	1.2	1.2	3.7	3.6	3.4	1.0	3.7
BatPV/Nycteris/DON007B018/BW/2021_B ⁺	33.5	24.0	21.0	22.8		3.2	3.2	3.1	3.2	3.3	3.1	3.3	0.6	3.1	3.2	3.3	3.8	3.6	2.1	3.3	3.7
BatPV/Nycteris/DON007B019/BW/2021 ²	32.9	1.8	19.2	0.6	22.2		0.8	3.0	3.1	1.0	3.0	3.1	3.2	3.0	1.0	1.0	3.7	3.6	3.4	1.0	3.7
BatPV/Nycteris/DON007B027/BW/2021	33.5	1.8	20.4	1.8	22.8	1.2		3.1	3.2	1.0	3.1	3.2	3.2	3.1	1.3	1.3	3.7	3.6	3.4	1.0	3.7
BatPV/Nycteris/DON007B031/BW/2021_A	30.5	21.0	1.2	19.8	21.0	19.2	20.4		0.8	3.1	0.0	1.3	3.1	0.0	3.1	3.1	3.7	3.6	3.2	3.1	3.7
BatPV/Nycteris/DON007B036/BW/2021	31.7	21.0	1.2	21.0	22.2	20.4	21.6	1.2		3.2	0.8	1.6	3.2	0.8	3.1	3.1	3.7	3.6	3.3	3.2	3.7
BatPV/Nycteris/DON007B037/BW/2021_A	32.9	1.8	21.0	1.2	24.0	1.8	1.8	21.0	22.2		3.1	3.2	3.3	3.1	1.4	1.4	3.7	3.6	3.4	1.0	3.7
BatPV/Nycteris/DON007B037/BW/2021_B	30.5	21.0	1.2	19.8	21.0	19.2	20.4	0.0	1.2	21.0		1.3	3.1	0.0	3.1	3.1	3.7	3.6	3.2	3.1	3.7
BatPV/Nycteris/DON007B042/BW/2021_A ⁺	32.9	22.8	4.2	21.6	24.0	21.0	22.2	3.0	4.2	22.8	3.0		3.3	1.3	3.2	3.2	3.8	3.6	3.4	3.2	3.7
BatPV/Nycteris/DON007B042/BW/2021_B ⁺	33.5	23.4	21.0	22.2	0.6	21.6	22.2	21.0	22.2	23.4	21.0	24.0		3.1	3.2	3.2	3.8	3.6	2.2	3.3	3.7
BatPV/Nycteris/DON007B048/BW/2021_A	30.5	21.0	1.2	19.8	21.0	19.2	20.4	0.0	1.2	21.0	0.0	3.0	21.0		3.1	3.1	3.7	3.6	3.2	3.1	3.7
BatPV/Nycteris/DON007B048/BW/2021_B	33.5	3.6	19.8	2.4	22.8	1.8	3.0	19.8	21.0	3.6	19.8	22.2	22.2	19.8		1.4	3.7	3.6	3.4	1.4	3.7
BatPV/Nycteris/DON007B051/BW/2021	34.1	2.4	19.2	2.4	23.4	1.8	3.0	20.4	20.4	3.6	20.4	22.2	22.8	20.4	3.6		3.7	3.6	3.4	1.4	3.7
BatPV/Rhinolophus/DON006B030/BW/2020 [†]	35.3	35.3	37.1	35.3	38.9	34.7	33.5	35.9	37.1	35.3	35.9	37.7	38.3	35.9	35.9	35.9		3.8	3.8	3.7	3.7
BatPV/Hipposideros/DON009B017/BW/2021 ³	30.5	31.7	31.1	32.3	32.3	32.3	32.3	30.5	31.1	32.3	30.5	32.9	31.7	30.5	32.9	32.3	40.1		3.7	3.6	3.5
BatPV/Nycteris/DON007B005/BW/2020 [†]	36.5	26.3	22.2	25.7	8.4	25.1	26.3	22.8	23.4	26.9	22.8	25.1	9.0	22.8	25.7	25.7	42.5	33.5		3.4	3.7
BatPV/Nycteris/DON007B007/BW/2020 ⁴	34.1	1.2	21.0	1.8	24.0	1.8	1.8	21.0	22.2	1.8	21.0	22.8	23.4	21.0	3.6	3.6	35.3	31.1	26.9		3.7
BatPV/Rhinolophus/DON010B009/BW/2021 ⁵	4.2	35.3	34.1	35.9	36.5	35.9	36.5	33.5	34.1	35.3	33.5	35.9	36.5	33.5	36.5	36.5	37.1	29.3	37.1	35.9	

Sequences within the same cluster:

¹ BatPV/Rhinolophus/DON010B023/BW/2021

² BatPV/Nycteris/DON007B020/BW/2021, BatPV/Nycteris/DON007B031/BW/2021_B, BatPV/Nycteris/DON007B034/BW/2021, BatPV/Nycteris/DON007B038/BW/2021, BatPV/Nycteris/DON007B044/BW/2021 and BatPV/Nycteris/DON007B049/BW/2021

³ BatPV/Hipposideros/DON009B025/BW/2021, BatPV/Hipposideros/DON009B018/BW/2021 and BatPV/Nycteris/DON010B024/BW/2021

⁴ BatPV/Nycteris/DON007B008/BW/2020; BatPV/Nycteris/DON007B010/BW/2020; BatPV/Nycteris/DON007B015/BW/2020; BatPV/Nycteris/DON007B021/BW/2020; BatPV/Nycteris/DON007B022/BW/2020; BatPV/Nycteris/DON007B023/BW/2020; BatPV/Nycteris/DON007B024/BW/2020; BatPV/Nycteris/DON007B025/BW/2020; BatPV/Nycteris/DON007B026/BW/2020; BatPV/Nycteris/DON007B028/BW/2020; BatPV/Nycteris/DON007B029/BW/2020; BatPV/Nycteris/DON007B030/BW/2020; BatPV/Nycteris/DON007B033/BW/2020; BatPV/Nycteris/DON007B035/BW/2020; BatPV/Nycteris/DON007B039/BW/2020; BatPV/Nycteris/DON007B040/BW/2020; BatPV/Nycteris/DON007B043/BW/2020; BatPV/Nycteris/DON007B047/BW/2020

⁵ BatPV/Hipposideros/DON009B008/BW/2021

* unique sequence