Surveillance for rabies-related lyssaviruses in South African insectivorous bat species

By

Colyn Stefan Grobler

Submitted in partial fulfilment of the requirements for the degree,
M.Sc in the Department of Medical Virology at the
Faculty of Health Sciences,
University of Pretoria
South Africa

January 2021

Supervisor and Co-supervisor

Professor Wanda Markotter and Dr Jessica Coertse

Biosurveillance and Ecology of Emerging Zoonoses Research Group

NRF-DSI South African Research Chair in Infectious Diseases of Animals (Zoonoses)

EXECUTIVE SUMMARY

Lyssaviruses are bullet shaped negative-sense RNA viruses that are all able to cause the fatal encephalitic disease known as rabies. The genus currently consists of 17 formally recognised viral species with one tentative species awaiting classification. The prototype virus for the *Lyssavirus* genus is the well-known rabies virus (RABV), while all other species in the genus are classified as rabies-related viruses. In South Africa specifically, RABV, Lagos bat virus (LBV), Duvenhage virus (DUVV), and Mokola virus (MOKV) are known to circulate, with RABV and DUVV associated with human fatalities. Active surveillance on rabies-related lyssaviruses in bats, specifically African insectivorous bat species, is either very sporadic or non-existing, providing an inaccurate overall representation of prevalence, diversity, and geographic distribution. Therefore, we conducted viral nucleic acid surveillance for lyssaviruses in different insectivorous bat species in South Africa. These samples were collected during routine field surveillance and included bats that were found dead, appeared to be displaying abnormal behaviour or taken as vouchers specimens as part of bat taxonomic studies. A quantitative real-time reverse transcription polymerase chain reaction assay, capable of detecting the diversity of lyssaviruses were used to test extracted RNA. Three brain samples tested positive and were further characterized by conventional RT-PCR, DNA sequencing and phylogenetic analyses targeting the nucleoprotein gene. One of the positive brains was detected from a Common slit-faced bat (*Nycteris thebaica*) and the other two positive brains were detected from the Natal long-fingered bat (*Miniopterus natalensis*). Phylogenetic analysis of the nucleoprotein indicated one detection to be a *Duvenhage lyssavirus* with the other two detections showing a close relationship with the *West Caucasian bat virus* species, previously only detected in Eastern Europe. However, a more than 20 % nucleotide divergence indicated it to be a potentially new lyssavirus species, Matlo bat lyssavirus. The virus was successfully isolated using the mouse inoculation test followed by full genome next generation amplicon sequencing. The results of the full genome characterisation further supported the initial findings with concatenated coding regions nucleotide divergence ranging between 16% and 23.7% as well as consistent phylogenetic tree topology groupings identical to initial phylogenetic analyses using multiple evolutionary models. The identification of a putative new lyssavirus highlights the importance of routine lyssavirus surveillance to understand the diversity. Further investigation is required to determine the possible reservoir species since the Natal long-fingered bats are known to co-roost with different bat species in caves. The potential of spillover to humans and other

animals is unknown but people often enter these bat roosts for traditional and recreational purposes and bats do come into contact with several animal species including humans during foraging.

Declaration

I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree M.Sc (Medical Virology) and the contained therein is my own work and has not been previously, in its entity or in its parts been submitted to any University for a degree.

Signature: **Date:**

24/11/2020

_____ _____

Acknowledgements

I would like to acknowledge the following:

Research funding:

This research was supported by the Cooperative Agreement Number (5 NU2GGH001874-02-00), funded by the Centers for Disease Control and Prevention. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention or the Department of Health and Human Services.

The National Research Foundation (NRF) is thanked for funding the equipment based at the DNA Sanger sequencing facility in the Faculty of Natural and Agricultural Sciences, University of Pretoria (**UID:78566**) that was used to generate Sanger sequencing and/or fragment analysis data presented in this work.

The South African Research Chair initiative (held by Professor Wanda Markotter) of the Department of Science and Inovation and National Research Foundation of South Africa for providing research funding.

The National Institute for Communicable Diseases (NICD) is thanked for the use of their Sequencing Core Facility that was used for next generation sequencing (NGS) and to generate NGS sequence data presented in this study.

Student funding:

In addition, student grant-linked funding supported by the South African Research Chair initiative (held by Professor Wanda Markotter) of the Department of Science and Inovation and National Research Foundation of South Africa (**Grant No 98339**).

Poliomyelitis Research Foundation (**Grant number: 18/54**)

Fieldwork, sample collection and research collaboration:

I would like to thank you all the students and colleagues of the Biosurveillance and Ecology of Emerging Zoonoses (BEEZ) Research Group in the Centre for Viral Zoonoses (University of Pretoria) for assistance in fieldwork to collect samples for this research project. I would also like to thank Dr. Teresa Kearney (Ditsong National Museum of Natural History) for bat species

morphological identification, AfricanBats (Mr Earnest Seamark) for assistance in collecting samples and training. Motjoli Resources, Aquila Steel, and Aquila Resources for the permission to conduct surveillance at Meletse as well as Motjoli Resources for granting us access to their property to conduct fieldwork research on the Madimatle/Gatkop Cave. I would also like to thank the Ga Mafefe community present in the Limpopo Province, for supporting this research as well as the bat rehabilitator community and bat interest groups for the submission of bats that were included or testing in this project.

Thank you to Dr Claude Sabeta for the use of the Onderstepoort Veterinary Research, Agricultural Research Council (ARC-OVR) facility and assistance during virus isolation.

I would also personally thank the following:

Professor Wanda Markotter- Thank you very much for providing me with the opportunity to pursue a career in science. Thank you for the guidance, advice and support you have given me during this degree. Thank you for providing me with funding to allow me to complete my degree as well as other additional opportunities such as conferences and additional training. Thank you for all your time and trust.

Dr Jessica Coertse- Thank you very much for the guidance, advice and support you have given me during this degree. Thank you for always being available when questions needed answering, troubleshooting was needed and for all your extra time and effort.

To my friends and colleagues at the University of Pretoria- Thank you for your support and additional guidance throughout my degree. Thank you all for making the difficult times a bit easier and always having advise and time to talk.

To my father and mother- My deepest gratitude and appreciation for all the love and support you have shown me through all the years of my studies and for granting me the opportunity to pursue this career. Thank you for always being there when things got hard and the endless amount of advice that I did not always listen to. Thank you for being the best parents any child could ask for.

Research outputs

Publications:

Coertse J, Grobler C, Markotter W. 2020. Genomic characterisation and taxonomic implications of a novel lyssavirus from insectivorous bats in South Africa (In preparation).

Coertse J, Grobler C, Sabeta C, Seamark E, Kearney T, Paweska J, Markotter W. 2020. Lyssaviruses in Insectivorous Bats, South Africa, 2003–2018 (Published).

Conference presentations:

Grobler C., Coertse J., Sabeta C., Markotter W. Discovery of a new rabies-related lyssavirus in South Africa. Virology Africa conference, 10-14 February 2020, Cape Town, South Africa (Oral presentation).

Grobler C., Coertse J., Markotter W. Discovery of a new rabies-related lyssavirus in South Africa. Faculty of Health Sciences Faculty day, 21 August 2019, Pretoria, South Africa (Oral presentation).

Table of Contents

List of Tables

List of Figures

Chapter 1 - Study Rationale

This dissertation consists of five chapters. In the first chapter, the rationale behind the research is briefly described. This includes a short introduction as well as the specific aims and objectives that the study set out to complete. Chapter two provides the necessary background information that pertains to the study in a literature review. Thereafter chapter three and four discuss the methodology, results and discussions of the respective surveillance and virus characterisation components of this project. Chapter five provides the conclusion and future perspectives of the study.

1.1 Brief background and rationale

Lyssaviruses, from the family *Rhabdoviridae*, are the causative agent of the fatal encephalitic disease known as rabies. Rabies virus (RABV) is classified as the prototype virus while all 16 other lyssaviruses in the genus are classified as rabies-related viruses (Amarasinghe et al., 2019). In Africa specifically, Lagos bat virus (LBV), Duvenhage virus (DUVV), Shimoni bat virus (SHIBV), Ikoma lyssavirus (IKOV) and Mokola virus (MOKV) are known to circulate, with LBV, DUVV and MOKV specifically identified in South Africa (Markotter and Coertse, 2018). Surveillance for rabies-related lyssaviruses is opportunistic and sporadic, providing overall an inaccurate representation of the actual epidemiology (Shipley et al., 2019; Markotter and Coertse, 2018; Rupprecht et al., 2011). Southern Africa is known to hosts multiple bat species, with South Africa specifically hosting atleast 56 identified species (ACR, 2019). Due to South Africa's diversity and widespread distribution of insectivorous bats species, it is importantthat we understand the prevalence and distribution of lyssaviruses in these bat species to understand epidemiology and potential pulic health implications.

1.2 Research aim and objectives

This study aims to conduct viral nucleic acid surveillance for lyssaviruses in insectivorous bats species in South Africa.

Specific objectives

- To test insectivorous bat brain samples collected in South Africa with a pan-lyssavirus qRT-PCR to detect lyssavirus RNA followed by DNA sequencing and phylogenetic analyses.
- To perform virus isolation from positive samples.
- To perform full-genome sequencing, genome characterization and phylogenetic analyses of positive samples.

Chapter 2 - Literature review

Introduction

Lyssaviruses are a group of viruses that are all capable of causing lethal encephalomyelitis in mammals, also known as rabies, with RABV responsible for the estimated death of 59.000 people each year (Amarasinghe et al., 2019; Hampson et al., 2015). The genus currently consists of 17 viral species recognised by the International Committee on Taxonomy of Viruses (ICTV), with one tentative species awaiting formal classification (Table 2.3). Lyssaviruses are present on all continents with the exception of a few islands and the polar regions. In the last decade, the number of described lyssavirus species has more than doubled which has led to intensified investigation into the lyssavirus genus (Markotter and Coertse, 2018; Dietzgen and Kuzmin, 2012b). In South Africa specifically, surveillance efforts and programs for rabies-related lyssaviruses are primarily opportunistic and often sporadic. This provides overall an inaccurate representation of the actual epidemiology and impact of lyssaviruses (Shipley et al., 2019; Markotter and Coertse, 2018; Rupprecht et al., 2011). In this study, we aimed to conduct viral nucleic acid surveillance for lyssaviruses in insectivorous bats species in South Africa. This chapter will be in the form of a literature review, discussing background information pertaining to the *Lyssavirus* genus, this study and relevant findings. Firstly, the lyssavirus genome structure, the specific genes and the protein functions will be discussed. Thereafter the taxonomic classification of lyssaviruses genus will be explained, followed by the description of lyssavirus species that have been implicated in Africa specifically. The host ranges of lyssavirus will also be discussed, followed by the pathogenicity in other mammals and in bats. The last section reviews lyssavirus surveillance within South Africa and current detection methods used.

2.1 Lyssavirus genome and protein functions

These viruses are bullet shaped with a negative, non-segmented single-stranded RNA genome and are known to have similar traits within the *Rhabdoviridae* family, including the virus morphology, general genome organisation, structural proteins, ribonucleoprotein (RNP) core and a helical nucleocapsid (Rupprecht and Nagarajan, 2014). The genome is approximately 12 kilobases (kb) long and encodes five structural proteins that are needed for virus replication. These proteins are the nucleo- (N), phospho- (P), matrix- (M), glyco- (G) and an RNA dependent polymerase (L) protein. Of these proteins, the N, M and L proteins have been found to be similar in structure and length between the different lyssavirus species, whereas the lengths of the P and the G have been found to vary more (Kuzmin et al., 2005). The 3' and 5' extremities of the genome, as well as short intergenic regions between the genes, regulate gene transcription as they contain binding sites for the polymerase enzyme, signals for the viral encapsidation as well as critical initiation sites for RNA synthesis. Each gene also contains a transcription initiation and termination signal as well as promotor sequences that initiates replication of the individual proteins (Rupprecht and Nagarajan, 2015; Arguin et al., 2002; Tordo et al., 1988).

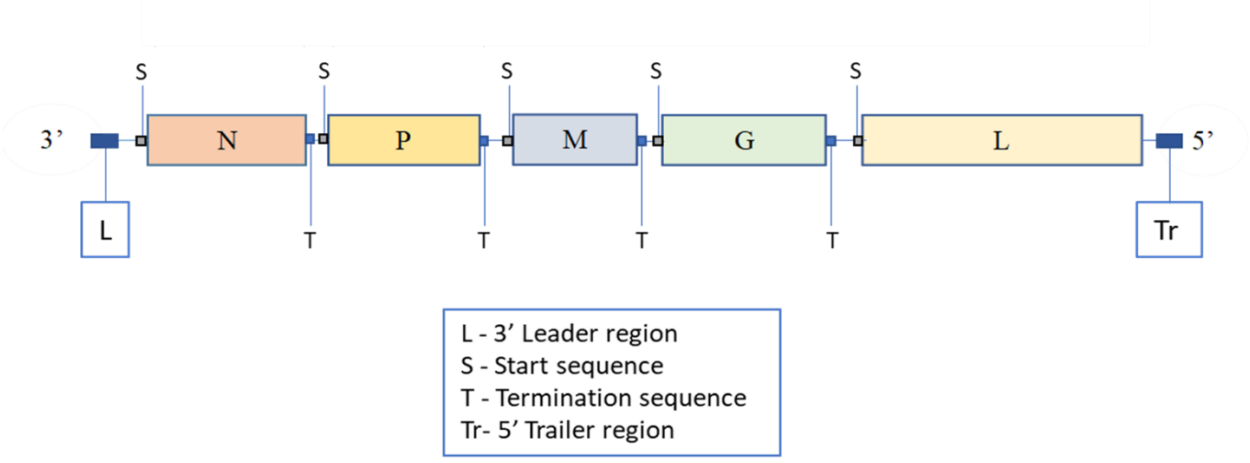


Figure 2.1 Schematic representation of the gene organisation of the lyssavirus genome, indicating the positioning and orientation of the five genes, the intergenic regions and the leader and trailer sequences. Image not drawn to scale. This is an adaptation of an original work "Laboratory techniques in rabies. Geneva: World Health Organization (WHO); 1996. Licence: ID: 352825". This adaptation was not created by WHO. WHO is not responsible for the content or accuracy of this adaptation. The original edition shall be the binding and authentic edition.

Nucleoprotein

The N protein is the most conserved of the 5 structural proteins. This allows for key functions that are dependent on protein-RNA genome interactions to be retained. This high level of nucleotide and amino acid conservation has led to the usage of N gene nucleotide identity as a quantitative measurement of lyssavirus classification. The prototype virus (RABV, Pasteur virus) contains 450 amino acids, with phosphorylation occurring at amino acid 389. The phosphorylation of amino acid 389 plays an integral role in genome assembly by stabilising N- and P- protein association (Mantari et al., 2019; Wunner and Conzelmann, 2013; Langevin et al., 2002).

Phosphoprotein

The P protein contains 297 amino acids (RABV, Pasteur) and is known to be the least conserved of the lyssavirus genes with the greatest variability being found between region 52-78 and 155-178 respectively (Wunner and Conzelmann, 2013; Marston et al., 2007). During the formation of progeny virus, the P protein (non-catalytic subunit) plays a vital role in binding the L protein (catalytic subunit) that allows for the formation of an active RNA polymerase complex. This P-L complex has been shown to direct the N protein to encapsidate the newly synthesised RNA (Jespersen et al., 2019; Albertini et al., 2011a; Albertini et al., 2008). These three proteins together with the genomic RNA form the ribonucleoprotein (RNP) complex. The P gene also contains a specific fragment proposed to be important for viral transport (Tan et al., 2007; Lo et al., 2001; Poisson et al., 2001). It was proposed that the peptide fragment located between amino acid 143 and 149 interacts with the LC8 dynein light chain through four specific amino acid residues that form the LC8 binding motif. Any mutation of one of these four amino acids would disrupt binding to the LC8 chain and would decrease transport of the virus from the peripheral site of infection to the central nervous system (CNS). Tan et al (2007) did however show that binding to the LC8 chain did not affect retrograde transport of the virus but that it did play an important role in viral replication and transcription (Jespersen et al., 2019; Tan et al., 2007). Notably, the P gene is translated from five in-frame start codons that result in the expression of the mentioned full-length P protein (P1) and then also in the expression of N-terminally truncated P proteins (tPs), that are designated the P2-, P3-, P4-, and P5 proteins. These tPs have been known to lack the L protein-binding domain, which indicates no cofactor activity. Importantly, the translation start codons of these tPs are conserved among RABV strains which suggests they have a critical role in RABV infection (Okada et al., 2016).

Matrix protein

The M protein is the smallest of the virion proteins with only 202 amino acids (RABV, Pasteur). It is responsible for the success of viral assembly as it is known to form the RNP-M protein complex that makes up the bullet shaped structure. The M protein also plays a major role in the budding of the newly formed virion from the cell after virion assembly (Luo et al., 2020; Wunner and Conzelmann, 2013; Mebatsion et al., 1996).

Glycoprotein

The G protein encodes for 524 amino acids (RABV, Pasteur) and is known to form four different domains surrounding the M protein and forms part of the viral envelope and forms trimer spikes. These domains are the signal peptide (19 amino acids), the ectodomain (439 amino acids), the transmembrane (22 amino acids) and the cytoplasmic domain (44 amino acids). The signal peptide is involved in the insertion of the G protein into the cell's endoplasmic reticulum but is missing from the mature G protein. The transmembrane domain incorporates the G protein into the virus envelope and the ectodomain plays an important role in the pathogenesis of the virus by interaction with host cell receptors (Coulon et al., 1998). The G protein of lyssaviruses contains a number of important domains that has been shown to play a crucial role in the pathogenesis during infection but also forms the trimer spikes protruding on the viral membrane that is a target or viral neutralising antibodies (Shipley et al., 2019). It has been shown, although only validated for RABV, that mutations of amino acid 333 present on the G protein ectodomain have resulted in less virulent strains when inoculating mice intra muscularly (Dietzschold et al., 1983). Along with amino acid 333, amino acid 330 has also been proven to reduce the pathogenicity especially when the mutation on amino acid 333 was also present (Coulon et al., 1998). Another important amino acid region on the G protein is Lys 194, which plays an important role in the internalization of the virus into cells (Faber et al., 2005). Although these studies have shown that amino acid 333 and 330 are important for viral pathogenesis, certain strains with these mutations have been shown to not be virulent, emphasizing they are not the only pathogenicity determining amino acid sites (Ito et al., 1994). When a non-virulent strain underwent mutations at Ser 242, Asn 255 and Leu 268 and replaced these amino acids with Ala 242, Asp 255 and Ile 268, the strain reverted back to virulence proving that multiple domains on the G protein influence pathogenicity (Takayama-Ito et al., 2006). The G protein has also been implicated in the successful binding of the virus into host cells. It has been reported that p75 neurotrophin receptor, neural cell adhesion molecule and

nicotinic acetylcholine receptor are important receptors for RABV binding, with specific amino acids being critical for successful viral attachment (Table 2.1).

Table 2.1 Amino acid positions on the glycoprotein of rabies virus shown to be important for mammalian receptor binding.

Receptor	Amino acid position	Reference
Nicotinic acetylcholine receptor	Amino acid region between 189 and 214	(Lentz et al., 1984)
P75 neurotrophin receptor	Phe 318 and His 352	(Tuffereau et al., 1998)
Neural cell adhesion molecule	Amino acids not yet determined	(Tuffereau et al., 1998)

The G protein is known to be the most important gene in pathogenicity, however Pulmanausahakul et al (2008) indicated that the other four proteins all play important roles in virus pathogenicityimportant region. When interchanging the G gene and M gene from a pathogenic strain into a non-pathogenic strain, the non-pathogenic strain reverted back to being pathogenic. When interchanging only the G gene, the non-pathogenic strain still reverted to being pathogenic, but to a lesser extent (Pulmanausahakul et al., 2008; Morimoto et al., 2000).

As previously mentioned, the lyssavirus G protein is present on the surface of the virus particle and is detected by the immune system to induce antibody formation. The specific antigenic sites on the G protein that have been mapped are listed in Table 2.2.

Table 2.2 Antigenic sites on the ectodomain of the glycoprotein of rabies virus, adapted from Kgaladi, 2015a.

Antigenic site	Amino acid position	References
I	231	(Dietzschold et al., 1983)
II	34-42 and 198-200	(Dietzschold et al., 1983)
III	330-338	(Dietzschold et al., 1983)
IV	263-264	(Ni et al., 1995)
A	343-434	(Benmansour et al., 1991)
Site not named	14-19	(Mansfield et al., 2004)
Site not named	251	(Lafay et al., 1996)

It has been shown that antigenic sites I and II are the most prominent when allowing cross-neutralisation of virus neutralising antibodies (VNA) against lyssaviruses from different phylogroups (Lafon et al., 1983). Some lyssavirus species, like those in the same phylogroup, have similar antigenic sites which provide them with a certain degree of similarity and possible cross-neutralisation by antibodies induced by a specific vaccine (Fisher et al., 2020; Shipley et al., 2019;

Klasse, 2014). All current rabies vaccines are based on RABV backbone and are effective against most phylogroup I lyssavirus but do not appear to have the same neutralizing effect on phylogroup II or non-grouped lyssaviruses (proposed phylogroup III) (Horton et al., 2014; Weyer et al., 2008; Hanlon et al., 2005; Nel et al., 2003).

RNA polymerase protein

The L protein is the largest of the lyssavirus proteins, encoding for 2142 amino acids (RABV, Pasteur) and constitutes more than half of the lyssavirus genome. The L protein forms the catalytic part of the polymerase complex (along with the noncatalytic P protein subunit), that is responsible for the greater part of enzymatic reactions which include viral RNA transcription, replication, 5' capping, 3' polyadenylation and transcript methylation (Rupprecht and Nagarajan, 2015; Barik et al., 1990; Tordo et al., 1988).

2.2 Lyssavirus taxonomy and classification

The genus *Lyssavirus* groups in the family *Rhabdoviridae* within the order *Mononegavirales*. As previously mentioned, there are currently 17 recognised viral species, with one tentative species awaiting formal classification (Table 2.3) (Kuhn et al., 2020). Currently, the species are formally grouped into two phylogroups based on genetic distances and serological cross-reactivity (Figure 2.2).

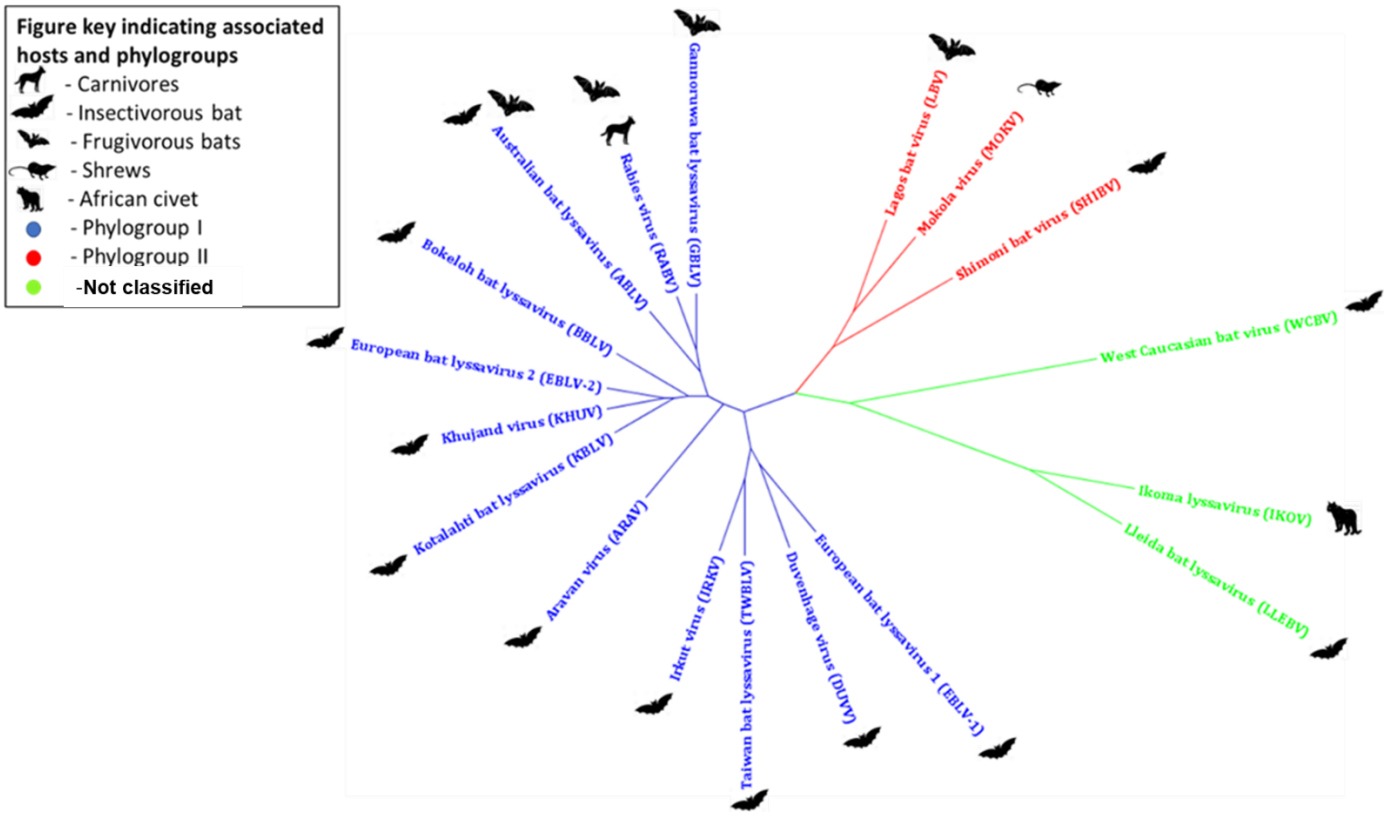


Figure 2.2 Bayesian phylogenetic analyses of lyssaviruses based on complete nucleotide sequences of the nucleoprotein gene (N), indicating all known lyssavirus species, phylogroups and the most commonly associated hosts.

Phylogroup I consists of *Rabies lyssavirus*, *Duvenhage lyssavirus*, *European bat 1 lyssavirus*, *European bat 2 lyssavirus*, *Australian bat lyssavirus*, *Aravanlyssavirus*, *Khujand lyssavirus*, *Irkut lyssavirus*, *Bokeloh bat lyssavirus*, *Gannoruwa bat lyssavirus*, *Taiwan bat lyssavirus* and the newly proposed species named Kotalahti bat lyssavirus. *Lagos bat lyssavirus*, *Mokola lyssavirus* and *Shimoni bat lyssavirus* are grouped into phylogroup II, while *West Caucasian bat lyssavirus*, *Ikoma lyssavirus* and *Lleida bat lyssavirus* are not formally classified into a phylogroup (Shipley et al., 2019; Markotter and Coertse, 2018)

Table 2.3 Rabies-related lyssaviruses associated with bats (Adapted from Shipley et el., 2019)

Lyssavirus	Continent	Country	Proposed bat host species	Spill over infections reported	Year first detected
Aravan virus (ARAV)	Asia	Kyrgyzstan	*Myotis blythi*	-	1991
Australian bat lyssavirus (ABLV)	Oceania	Australia	*Pteropus alecto*	Horses, Human	1996
Bokeloh bat lyssavirus (BBLV)	Europe	Germany, France	*Myotis nattereri*	-	2010
Duvenhage virus (DUVV)	Africa	South Africa, Kenya and Zimbabwe	*Nycteris thebaica*	Human	1971
European bat Lyssavirus 1 (EBLV-1)	Europe	France, Germany, and Spain	*Eptesicus serotinus*	Stone marten, Cats, Sheep, Human	1968
European bat lyssavirus 2 (EBLV-2)	Europe	The Netherlands, Switzerland, UK, France, Germany, Luxembourg and Finland	*Myotis daubentoniid, Myotis dasycneme*	Human	1996
Gannoruwa bat lyssavirus (GBLV)	Asia	Sri Lanka	*Pteropus medius*	-	2014
Ikoma lyssavirus (IKOV)**	Africa	Tanzania	N/A	Civet	2009
Irkut virus (IRKV)	Asia	Russian Federation and China	*Murina leucogaster*	Dog, Human	2002
Kotolahti Bat Lyssavirus (KBLV)*	Europe	Finland	*Myotis brandti*	-	2017
Khujand virus (KHUV)	Asia	Tajikistan	*Myotis mystacinus*	-	2001
Lagos bat virus (LBV)	Africa	Nigeria, Senegal, Ghana, Kenya, Central African Republic and South Africa	Various species of frugivorous bats	Cats, Dogs, Mongoose	1956
Lleida bat lyssavirus (LLEBV)	Europe	Spain and France	*Miniopterus schreibersii*	-	2011
Mokola virus (MOKV)***	Africa	Nigeria, Zimbabwe, South Africa, Ethiopia	N/A	Cats, Dogs, Human	1968
Shimoni bat virus (SHIBV)	Africa	Kenya	*Macronycteris vittatus*	-	2009
Taiwan bat lyssavirus (TBLV)	Asia	Taiwan	*Pipistrellus abramus*	-	2016
West Caucasian bat virus (WCBV)	Europe	Russian Federation, Italy	*Miniopterus schreibersii*	-	2002

*Virus awaiting formal classification.

**Virus never detected in a bat species, only considered a potential bat virus.

***Virus not detected in a bar species.

Specific lyssavirus species demarcation criteria have been proposed by the ICTV when species classification is considered. These demarcation criteria have been

introduced in order to best reflect the evolutionary as well as ecological relationships of these viruses. Up to 1956, it was suggested that the prototype virus, RABV was antigenically unique. It was only after the discovery of DUVV, LBV and MOKV during the late 1950s to 1970s, that the lyssavirus genus was subdivided into four serotypes (Rabies virus, Lagos bat virus, Mokola bat virus and Duvenhage virus), based on specific serological cross reactivity as well as monoclonal antibody typing (Meredith et al., 1971; Shope et al., 1970; Boulger and Porterfield, 1958a).

Initially, the discovery of EBLV-1 and EBLV-2 led to the formation of serotype 5. Shortly thereafter the development of polymerase chain reaction (PCR) and subsequent nucleic acid sequencing, allowed for genetic comparison of specifically the conserved full lyssavirus N gene. It was proposed that the serotypes should be divided into seven genotypes with a new lyssavirus genotype defined as more than 80% nucleotide differences and also more than 93% amino acid differences of the full N gene (Nadin-Davis and Real, 2011; Bourhy et al., 1993). The genotype classification criteria were based on three main reasons. The N gene was specifically chosen to evaluate the performance of the antigenic and genetic tools that were used for initial virus classification when comparing their reactivity with antinucleocapsid monoclonal antibodies. The N gene is well conserved in all lyssaviruses it allows for a clear division between lyssavirus genotypes across relatively long evolution patterns (Markotter et al., 2008a; Bourhy et al., 1993). The genotypes were then divided into two immunopathologically as well as genetically distinct phylogroups with phylogroup I including genotypes RABV, DUVV, EBLV-1, EBLV-2, ABLV, and phylogroup II including genotypes MOKV and LBV (Badrane and Tordo, 2001). However, with the discovery of ARAV and KHUV as well as WCBV, it was recommended that the genotype demarcation criteria be reassessed (Kuzmin et al., 2003). It was subsequently suggested that the criteria of lyssavirus species classification take into consideration genetic distance of the complete N gene with a threshold of between 80-82% nucleotide identity or 80-81% nucleotide identity for the concatenated coding regions of all five lyssavirus genes, although the identification and formation of specific viral clusters within the LBV species have highlighted shortcomings when specific intra- and inter- genotypic identities begin to overlap when comparing genes other than the N gene (Markotter et al., 2008a). The use of criteria such as host range and geographical distribution was also suggested, however at the time, such information regarding the natural history of lyssaviruses was restricted (Kuzmin et al., 2005).

Recent taxonomic classification changes implemented by the ICTV require there be consistency in the phylogenetic trees obtained when using multiple evolutionary models. The antigenic

reaction patterns between the virus and anti-nucleocapsid monoclonal antibodies as well as the cross-reactivity obtained during serology when using monoclonal antisera should also be considered. This, along with any additional characteristics such as any ecological properties, host or geographical ranges as well pathological features can also be used to demarcate species identity (Rupprecht et al., 2017). In 2016, the ICTV also adopted the partial appropriation of binomial species nomenclature that allowed for the classified genus name to appear after the species name (Afonso et al., 2016).

Currently, the ICTV have established specific criteria for the classification of different lyssavirus species and viruses assigned to different species must have several of the determined characteristics (Kuhn et al., 2020; ICTV, 2020). There must be a threshold of 80–82% nucleotide identity for the complete N gene or 80% nucleotide identity for concatenated coding regions of N-, P-, M-, G-, and L gene. Phylogenetic trees based on the entire N, or G, or L gene, or concatenated coding sequences of the N-, P-, M-, G-, and L gene, the new viral species must not represent a sister branch to a virus from an established species. It must be placed ancestrally to a group (cluster) of phylogenetically related viruses that belong to several established species. The new viral species must be distinguished serologically in virus-neutralization tests and also occupy a distinct ecologic niche as evidenced by host species, pathobiological properties, or geographical range (ICTV, 2020).

2.3 Lyssavirus species

Rabies lyssavirus

This is the type species of the *Lyssavirus* genus and includes the sole representative, rabies virus (RABV), which is associated predominantly with carnivores and is not found in bats on the African continent to date (Markotter et al., 2008b). The host range of RABV has been linked to specific geographical range, causing the formation of specific phylogenetic different clades. The geographical distribution of these clades has shown RABV to only circulate in bats in the Americas. Currently, RABV is still responsible for the majority of human lyssavirus infections worldwide and all available vaccine strains used for both humans as well as veterinary applications are based on RABV (Rupprecht et al., 2018). The epidemiological study of RABV has shown dogs to be the major host in most parts of the world (Suleiman et al., 2020). With canine rabies occurring throughout the year in endemic regions (Suleiman et al., 2020). In Africa specifically, the RABVs have been sub-divided into four lineages. These lineages are Africa 1a (which are known to be

primarily restricted to North and West Africa), 1b (South-East Africa), 2 (this lineage includes wild-type strains from western African countries) and 3 and 4 that are related and part to the cosmopolitan lineages respectfully (Sabeta et al., 2020; David et al., 2007). These lineages are known to be separated geographically as well as to be host specific (Sabeta et al., 2020). In South Africa, there are two major variants of RABV that are distinguished and well described (Weyer et al., 2011). These variants are known to circulate within the Canidae and Herpestidae species (Weyer et al., 2011; Nel et al., 2005). The canid RABV variant is widespread in South Africa and is mainly associated with the domestic dog (*Canis familiaris*). The other south African variant is known as the mongoose RABV variant and circulates in multiple herpestids scattered over the central region of South Africa (Sabeta et al., 2020; Weyer et al., 2011, Nel et al., 2005, Coetzer et al., 1995).

Lagos bat lyssavirus

The species is represented by Lagos bat virud (LBV) which is a uniquely African virus and was first isolated in the early 1950s from an African straw-coloured fruit bat (*Eidolon helvum*). Although initially described as different to RABV it was only classified as a new lyssavirus species in 1970 based on specific complement fixation and neutralization tests results (Shope et al., 1970; Boulger and Porterfield, 1958a). Thereafter multiple isolations were made from several frugivorous bat species that include the Wahlberg's epauletted fruit bat (*Epomophorus wahlbergi*), the Egyptian rousette bat (*Rousettus aegyptiacus*), as well as a single isolate reported from Peters's dwarf epauletted fruit bat (*Micropterus pusillus*) and one isolation reported from the insectivorous Gambian slit-faced bat (*Nycteris gambiensis*) (Freuling et al., 2015; Kuzmin et al., 2010, Kuzmin et al., 2008; Markotter et al., 2008a**)**. Spillover infections have been reported in dogs, a cat, as well as a mongoose (Markotter et al., 2020; Markotter and Coertse, 2018; Kuzmin et al., 2008b; Kuzmin et al., 2008a; Markotter et al., 2008b; Markotter et al., 2008a; Markotter et al., 2006; Foggin, 1988). It was also established that there is great nucleotide diversity (up to 23.7%) between different isolates of LBV detected in different geographical locations across Africa (Kuzmin et al., 2010; Markotter et al., 2008a). After further characterisation of the different isolates, LBV was divided into four clades/lineages namely clade A-D, all displaying the highest intrinsic diversity of all lyssavirus species. The clades also show association with specific the distribution of bat species as well as geographical locations (Markotter and Coertse, 2018; Delmas et al., 2008; Markotter et al., 2008a). Vaccine protection data suggests the commercially available vaccine provides little to no

protection against LBV as it phylogenetically groups as a Phylogroup II lyssavirus (Weyer et al., 2008; Hanlon et al., 2005).

Mokola lyssavirus

The species is represented by Mokola virus (MOKV) which was initially isolated from wild shrews (*Crocidura flavescens*) in Nigeria in 1968. After serological tests indicated antigenetic cross-reactivity with LBV and even with RABV, the virus was believed to be rabies-related, and further established as an indepemdemt lyssavirus serotype (Kemp et al., 1972; Shope et al., 1970). After the initial isolation, there were reports of MOKV infection in two children. These reports can unfortunately not be confirmed as the specimens obtained from the children are not available and no genetic characterisation was performed (Familusi et al., 1972). Since the initial MOKV identification, the virus has been identified in dogs, cats and shrews (Markotter and Coertse, 2018; Sabeta et al., 2007; Meredith and Nel, 1996; Saluzzo et al., 1984; Foggin, 1983; Kemp et al., 1972) but currently the reservoir host for MOKV is unknown. There has been data suggesting the presence of MOKV virus neutralising antibodies (VNA) in frugivorous bats but due to cross-reactivity between MOKV and LBV (also isolated in frugivorous bats), the circulation of MOKV in bats could not be confirmed to date (Kgaladi et al., 2013).

Duvenhage virus

The species is represented by Duvenhage virus (DUVV) which has only been isolated five times with three of these originatingfrom fatal human infections after exposure to unidentified bats. *N. thebaica* has thus far been the only host species linked to infections after confirmed taxonomic identification. The first isolation of DUVV was made from a human in 1970, which was thought to have been exposed to a Common bent-wing bat (*Miniopterus schreibersii)* (name changed to Natal long-fingered bat (*Miniopterus natalensis*) (ACR, 2019; Miller-Butterworth et al., 2005; Appleton et al., 2004; Meredith et al., 1971). The bat species was only implicated based on abundant presence in the area. The second isolation of DUVV in 1981 was also from what was believed to be an insectivorous bat with no confirmation of species identification (Van der Merwe, 1982). The third isolation was made from a *N. thebaica* in 1986 in Bulawayo, Zimbabwe which was the first definitive host species confirmation for DUVV infection (Foggin, 1988). The fourth and fifth isolations of DUVV were both made from infected humans in Pilanesberg area (South Africa) and Tsavo West (Kenya) respectively (Van Thiel et al, 2009, Paweska et al., 2006). Unfortunately, the bats implicated in these cases were never captured or positively identified but

were believed to be insectivorous bats due to their reported small size. All isolations, with the exception of the Kenyan case, have been geographically obtained from the northern regions of South Africa and the southern part of Zimbabwe. When the genetic divergence between the isolates are compared, two distinct groups are formed with the Kenyan isolate clearly grouping separate from the southern African isolates (Koraka et al., 2012; Van Thiel et al, 2009).

Ikoma lyssavirus

In 2009, IKOV was isolated for the first and only time, from an African civet (*Civettictis civetta*). The civet displayed clinical signs typical to rabies and was killed by field rangers in the Serengeti National Park after an unprovoked attack on a child. Initially, lyssavirus RNA was detected from the civet brain material and after further characterisation, IKOV was successfully isolated in 4-week-old mice (Horton et al., 2014; Marston et al., 2012). Based on genetic characterization, it was established that the isolate represents a new lyssavirus species. The nucleotide sequence data showed between 61% to 62.1% sequence identity compared to the other lyssavirus whole-genome sequences, being most closely related to WCBV (Marston et al., 2012). This case in a civet was seen as a spillover infection caused by a novel lyssavirus with an unknown reservoir host and hypothesized to be bat borne due to its phylogenetic relatedness to WCBV and LLEBV (Rupprecht et al., 2017; Horton et al., 2014).

Shimoni bat virus

In 2009, Shimoni bat virus (SHIBV) was isolated for the first and only time from the brain of a striped leaf-nosed bat (*Macronycteris vittatus*) (previously *Hipposideros commersoni*), found dead in a cave in the coastal region of Kenya (Foley et al., 2017; Kuzmin et al., 2010). Genetic distances after analysing the full genome suggested that this virus should be included into a new lyssavirus species related phylogenetically to *Lagos bat lyssavirus* and *Mokola lyssavirus* as a member of Phylogroup II(Kuzmin et al., 2010). The identification of *M. vittatus* as the reservoir host for SHIBV was further supported by a study conducted in Kenya where multiple bat species were sampled and tested for the presence of SHIBV VNA. *M. vittatus* showed significantly higher VNA titers against SHIBV when compared to other bat species included in the study (Kuzmin et al., 2011).

European bat 1 lyssavirus and European bat 2 lyssavirus

European bat lyssaviruses were originally believed to be related to DUVV based on initial monoclonal antibody typing (Schneider, 1982). After their genes were sequenced and analysed, two new biotypes were initially identified, and they were subsequently designated as EBLV-1 with EBLV-2. Both EBLV-1 and EBLV-2 were later identified as distinct genotypes and as further different species based on ICTV requirements. The reservoir host for EBLV-1 is considered to be the Serotine bat (*Eptesicus serotinus)* but it has occasionally been associated with other insectivorous bats as well as spill-over infections were documented in sheep, domesticated cats and a stone marten (Eggerbauer et al., 2017). EBLV-2 has been most frequently linked to Daubenton's bats (*Mytois daubentonii*) and also pond bats (*Myotis* dasycneme) (McElhinney et al., 2018). Unlike EBLV-1, there have not been any implications of spill-over events into other wildlife for EBLV-2, but both EBLV-1 and EBVL-2 have been implicated in the death of humans (Markotter and Coertse, 2018; McElhinney et al., 2018, Fooks et al., 2003; Bourhy et al., 1992; Lumio et al., 1986).

Bokeloh bat lyssavirus

BBLV was initially isolated from a Natterer's bat (*Myotis nattereri*) in 2010 in Germany after the bat was caught and kept in captivity where it started displaying signs of illness (Freuling et al., 2011). After the bat died, nucleic acid and antigenic analyses revealed the virus was most closely related to phylogroup I lyssaviruses, particularly to Khujand virus (KHUV). Since the initial isolation, there have been five additional BBLV isolations in Germany, two isolations in France as well as a more recent isolation in Poland (Markotter and Coertse, 2018; Smreczak et al., 2018; Nolden et al., 2014; Freuling et al., 2013; Picard-Meyer et al., 2013). Currently, all but one isolation were made from *M nattereri*, leading to the identification of this insectivorous bat as the potential host for BBLV (Markotter and Coertse, 2018; Smreczak et al., 2018; Nolden et al., 2014; Freuling et al., 2013; Picard-Meyer et al., 2013).

Lleida bat lyssavirus

LLBV was first discovered in the City of Lleida in Spain in 2011. The virus was identified from *M. schreibersii* and did not belong to either Phylogroup I or Phylogroup II lyssaviruses. Instead, the virus proved to be most closely related to IKOV based on partial N gene sequence identity and later using full genome sequence comparison (Marston et al., 2017; Ceballos et al., 2013). In 2017, a second isolation of LLBV was also made from a *M. schreibersii* bat found dead in an

underground site by a bat specialist (Picard-Meyer et al., 2019). The identification of LLBV in *M. schreibersii*, on two separate occasions, is indicative of this bat being the potential host for this lyssavirus.

Australian bat lyssavirus

ABLV was first isolated in Australia in 1996 from a black flying fox (*Pteropus alecto*) after Australia was thought to be rabies free. Since the initial discovery, ABLV has been isolated from at least four species of the *Pteropus* spp. bats as well as from the insectivorous bat *Saccolaimus albiventris*; both the pterodid and insectivorous bat variants of ABLV caused human rabies cases (Francis et al., 2014; McColl et al., 2000; Hanna et al., 2000; Hooper et al., 1997; Allworth et al., 1996). Subsequently, there have also been two cases of cross species transmission in horses (Annand and Reid, 2014). Further molecular characterisation has subsequently identified two separate variants present in ABLV, one variant being specifically associated with the fruit bat species and the other with the insectivorous bat species (Markotter and Coertse, 2018; Guyatt et al., 2003).

Aravan lyssavirus, Khujand lyssavirus and Irkut lyssavirus

Aravan virus (ARAV) was originally isolated in 1991 in Kyrgyzstan from the brain of a lesser mouse-eared bat (*Myotis blythi)* while Khujand virus (KHUV) was isolated in 2001 in the northern Tajikstan from a whiskered bat (*Myotis mystacinus*) (Kuzmin et al., 2003). Irkut virus (IRKV) was first isolated from a greater tube-nosed bat (*Murina leucogaster*) in 2002 in Russia and also more recently in the same bat species in the Jilin province in People's Republic of China (Liu et al., 2013; Botvinkin et al., 2003). From the above-mentioned viruses, IRKV is the only virus that has been associated with human fatalities after causing the death of a 20-year girl in the Primorye Territory of Russia (Leonova et al., 2009). Recently, a case of IRKV was also detected in a dead dog, previously associated with a human bite case. Further pathogenicity studies proved that IRKV can cause rabies in cats and dogs (Teng et al., 2018).

West Caucasian bat lyssavirus

WCBV was first isolated in Russia in 2002 from the brain of a *M. schreibersii* bat. At that moment, it was confirmed to be the most divergent lyssavirus, segregated in a new species, and could not be associated with either Phylogroup I or II (Kuzmin et al., 2008b; Botvinkin et al., 2003). In a study conducted in Kenya, WCBV neutralizing antibodies were found in *Miniopterus* spp bats

sampled across four different locations. The researchers could, however, not isolate any virus from the sampled bats but the seroprevalence may reflect past exposures indicating the possible presence of WCBV or a related virus in their populations (Kuzmin et al., 2008b). In June of 2020, a lyssavirus that showed 98,52% sequence similarity to WCBV was isolated from a cat in Arezzo, Italy. The cat showed neurological symptoms and eventually died. Early reports of bat colonies near the house in which the cat lived are suggested to be the source of the virus (Coxon et al., 2020).

Taiwan bat lyssavirus

TWBLV was first identified in two Japanese pipistrelles bats (*Pipistrellus abramus*) in Taiwan in 2016 and 2017. The virus was determined to be a member of Phylogroup I lyssaviruses, most closely related to EBLV-1, IRKV, and DUVV (Hu et al., 2018). In 2020 TWBLV was officially classified as a new lyssavirus species by the ICTV (Kuhn et al., 2020). To date, no human cases have been reported for TWBLV.

Gannoruwa bat lyssavirus

GBLV was first isolated in 2015 in Sri Lanka from an Indian flying fox (*Pteropus medius)*, previously known as *Pteropus giganteus* before taxonomic revision of the species classification. GBLV was shown to be a member of Phylogroup I lyssaviruses. Full genome sequence analyses showed GBLV to be the closest related to RABV and was classified as a new *lyssavirus* species (Gunawardena et al., 2016).

2.4 Bats as viral reservoirs

Bats, order Chiroptera, account for 20% of all extant species today and is the second most speciated mammalian order (Markotter et al., 2020; Simmons et al., 2008; Wilson and Reeder, 2005). The order can be divided into two suborders namely the Vespertilioniformes and Pteropodiformes with frugivorous being included in the latter (Markotter et al., 2020: Hutcheon and Kirsch, 2006; Teeling et al., 2005). Their dietary requirements can broadly be summarised as insectivorous, frugivorous, carnivorous, omnivorous and sanguivorous with Africa boasting species from the former four groups. Bats are also known to have a very diverse biology and ecology that often present unique characteristics that allow them to become the hosts of a variety of infectious agents. Bats are known to have exceptionally long-life spans compared to other mammals of the same body size (Salmon et al., 2009). With the increased life span, bats host an environment allowing

for sustainable viral replication and virus survival (Olival et al., 2017). As bats can colonize roosts from groups of less than 10 to more than 200 000 individuals during seasonal breeding, viruses are transmitted between infected and non-infected members due to some natural behaviour factors, such as biting during mating and grooming with possible airborne transmission also being implicated, which can cause increased transmission (Plowright et al., 2015; Wong et al., 2007; Winkler et al., 1973). Bats are known to roost in caves, rock crevices, in tree trunks or on tree branches. They also occupy a wide variety of man-made constructions such as buildings, bridges, mines and tombs that often leads to a higher likelihood of human and animal interaction with subsequent pathogen transmission (Markotter et al., 2020). Choice of roosts is influenced by the specific bat species, the current season as well as the sex of the individuals (Luis et al., 2015).

Bats are also the only mammal capable of true flight. This allows for the fast movement of bats long distances, with the Egyptian flying foxes being recorded to migrate as far as 500 km and the straw-coloured fruit bat (*Eidolon helvum*) involved in continent-wide panmixia of zoonotic viruses (Peel et al., 2013; Jacobsen and Du Plessis, 1976). Bats are also known to have unique immunology traits (Schountz et al., 2017). Genome studies have revealed the positive evolution of the mitochondrial DNA damage and repair pathways along with the evolution of self-powered flight (O'shea et al., 2014; Zhang et al., 2013). Self-powered flight is believed to causes an increase in metabolic rate in bats, thus increasing body temperature that was suggested as a viral control mechanism. It has also been suggested that bats have higher amounts of naïve immunoglobulin groups in circulation as well as possess a diversity of immunoglobulin segments. The differences between bat and human immune response in the event of viral infection have been well documented and it is believed that these differences allow for a quicker and more effective immune response to viral infection (Schountz et al., 2017; Kuzmin et al., 2017; Zhou et al., 2016; Zhou et al., 2014; Zhang et al., 2013; Baker et al., 2013; Zhou et al., 2011). These abilities do allow for the increased potential of virus transmission due to possible contact with other animals (Kuzmin and Rupprecht, 2015; Wang et al., 2011).

2.5 Lyssavirus pathobiolgy

The host range for lyssaviruses varies widely as all mammals can be infected with rabies, but bats are the most prominent hosts as all lyssaviruses, probably except IKOV and MOKV, are associated with bats (Hayman et al., 2016; Horton et al., 2014; Badrane and Tordo, 2001). Lyssaviruses are neurotropic pathogens that enter the body through contact of infected saliva with an open wound, caused by a bite or scratch from a rabid host. The virus may undergo several cycles of replication

at the entry site before entering neurons through the neuromuscular junction (Charlton et al., 1997). After the virus has attached to its specific receptor it enters the cell through endocytosis, and then transported through retrograde axonal flow (Albertini et al., 2011a). The pH in the endosome decreases causing fusion of the endosome membrane and viral membrane, and viral nucleocapsid is released into the cytoplasm. The negative-sense viral RNA is transcribed by the viral RNA-dependent RNA polymerase (RDRP) that allows the further transcription of the monocistronic (+) RNAs for the five mRNAs that all but one produce a single protein and the whole-length positive-sense viral antigenome (Albertini et al., 2011a). After viral replication and assemblage are complete, the viral progeny is then released at the neurons' synaptic junctions. Once in large nerves and further in the central nervous system (CNS), the virus is shielded from the host immune system surveillance. An average asymptomatic incubation period of 3-4 weeks but can vary from months to years (Boland et al., 2014). After the virus reaches the CNS, it spreads quickly and infects almost all parts of the CNS with the medulla oblongata being the principal site for virus detection in diagnostic tests. After further viral replication, the virus speads via neurons to other organs including salivary glands to ensure transmission to a susceptible host (Hanlon et al., 1999). The disease progresses quickly, and death usually occurs within five to seven days after onset of clinical symptoms (Willoughby Jr et al., 2005). During the asymptomatic period (time from infection to onset of symptoms), there is no laboratory diagnosis to detect the virus, due to the lack of a prominent immune response (Kgaladi et al., 2017) and the virus being present inside neurons (Charlton et al., 1997). In a mechanism not yet understood, most bats are able to tolerate infection to a point where a potential abortive infection (an infection in which some or all viral components are synthesized but no infectious virus is produced, and disease does not occur) and formation of lyssavirus neutralising antibody takes place which makes them ideal hosts (Plowright et al., 2015; Tjørnehøj et al., 2006). However, in a small percentage of bats, lyssavirus infection progresses leading to the proliferation of the virus in the central nervous system and ultimately the brain, leading to further dissemination in other organs and also saliva. These infections can ultimately lead to exposure of humans and other animals but are however rare as demonstrated by previous studies that found infection rates between 0 and 1 % in specific bat species (Blackwood et al., 2013; Constantine, 1967).

Various infection studies with lyssaviruses have been performed providing data supporting the claim of bats being able to tolerate infection to a certain extend (Banyard et al., 2011). Infection studies using EBLV-2 with *M. daubentonii* showed that direct intracranial inoculation leads to the development of rabies but inoculation via other routes, such as intramuscular did not lead to

infection or led to the seroconversion of the bats challenged (Johnson et al., 2008). Intracranial inoculation of EBLV-1 into the brains of *R. aegyptiacus* caused disease and death in 63% of the inoculated animals, although surprisingly not in all inoculated individuals (Freuling et al., 2009; Van der Poel et al., 2000). Another study using the experimental infection of Great fruit-eating bats (*Artibeus intermedius*) was performed with RABV. In this study, three doses of RABV and three different routes of infection were used and only one out of 35 bats died of rabies without showing any clinical signs (Obregón-Morales et al., 2017). Such experimental infection studies have highlighted the importance that the route of infection, viral species, viral dosage and dependence of the virus on the specific hostin lyssavirus pathogenesis and the outcome of infection (Banyard et al., 2011).

2.6 Lyssavirus surveillance

Lyssavirus surveillance has described several new lyssaviruses in the past decade. Up to the end of the 20th century, only seven lyssavirus species were established. Since then, 11 new species have been added to the *Lyssavirus* genus, mainly attributed to the increase in surveillance (Shipley et al., 2019; Markotter and Coertse, 2018). The overall diversity, epidemiology and public health burden of rabies and rabies-related lyssaviruses are underestimated (Shipley et al., 2019; Markotter and Coertse, 2018; Banyard and Fooks, 2017; Hampson et al., 2015). Although the majority of human deaths are attributed to RABV, the actual impact of the rabies-related viruses is not known. In the cases where RABV diagnostics are performed, the methods used are frequently not able to discriminate between different lyssaviruses due to cross-reaction between closely related lyssavirus. An example of this could be seen in the identification of IKOV, where initial diagnostic screening did not identify the presence of a novel virus and only after further molecular characterisation, was the isolate determined to be a novel lyssavirus (Horton et al., 2014; Marston et al., 2012). Another obstacle that is often faced when reporting on the actual impact of the rabies-related viruses, is once off isolations of novel lyssavirus (only one isolation was made), often causing limited opportunities for follow up studies and a lack of characterization of novel viruses (Markotter and Coertse, 2018). To improve our understanding of lyssavirus diversity, geographical distribution and potential host range, improved surveillance in bats is necessary. The surveillance strategies can be focused on detecting lyssavirus specific VNA, lyssavirus antigen or viral RNA, with follow up virus isolation and full genome characterization.

2.6.1 Serological surveilance

The main approach of serological surveillance is to detect virus neutralizing antibodies against a specific lyssavirus in serum collected. The presence of such antibodies indicates previous exposure to a specific (or closely related) virus or very late stage of clinical infection (Banyard et al., 2014). Virus neutralization tests are the gold standards including the Rapid fluorescent focus inhibition tests (RFFIT) (Smith et al., 1973 Kuzmin et al., 2008a) and the Fluorescent antibody virus neutralisation (FAVN) (Trimarchi et al., 1996). Serum is tested by adding a known amount of specific challenge virus to it. If antibodies that are able to neutralise the specific or closely related virus being tested for are present in the serum sample, they will bind and neutralise the virus. When cells are then added, infection is blocked and when a fluorescein labelled conjugate specific to the virus is added, the amount of neutralisation can be reported by the presence of infected cells.

As described before, bats are able to tolerate and clear infection that results in an immune response to the virus (Baker et al., 2013). This does cause the seroprevalence of lyssaviruses identified in bats to be significantly higher when compared to the detection rate of virus (<1%) (Markotter and Coertse, 2018; Rupprecht et al., 2011). The presence of VNA in bat populations provide an insight into the possible lyssavirus diversity present in the specific bat species as well as the geographical location, but these studies will however not be able to identify exact virus due to cross-reaction between closely related lyssaviruses. The use of various methods, different challenge viruses, variation in cut-off values and species cross-neutralisation does warrant that serological data be interpreted with caution (Markotter and Coertse, 2018).

2.6.2 Lyssavirus antigen and viral RNA detection

The detection of lyssavirus antigen is primarily achieved using the gold standard diagnostic tool, the fluorescent antibody test (FAT). The test that was first developed in 1959 and then later modified, is currently the recommended method by the World Health Organization for rabies diagnosis (Dean et al., 1996; Goldwasser et al., 1959). The FAT detects viral antigen present in brain material by immunofluorescence when slide with a fixed brain impression is treated with polyclonal anti-nucleocapsid antibodies tagged with fluorescein isothiocyanate (FITC) (Dean et al., 1996). Lyssavirus species specific monoclonal antibodies can also be used, to distinguish between specific viruses (Coetzer et al., 2014; Ngoepe et al., 2014; Markotter et al., 2009). With the development of molecular techniques and thus the ability to distinguish viruses according to genetic differences, the polymerase chain reactions (PCR) is widely used as a detection tool with follow up DNA sequencing to characterize the virus (Marston et al., 2019; Wang et al., 2019;

Nadin-Davis and Real, 2011; Coertse et al., 2010; Sacramento et al., 1991). In recent years, further development and use of the conventional PCR, hemi-nested PCR and real-time PCR revolutionised the surveillance and ability of these assays to detect and characterise a diversity of known and unknown lyssaviruses (Fisher et al., 2020; Coertse et al., 2019; Gigante et al., 2018; Wadhwa et al., 2017). Real-time PCR platform allows for monitoring in real time using fluorescent probes or intercalating dyes (Coertse et al., 2010). The development of probes and primers able to detect the diversity of known lyssaviruses, as well as other advantages such as high sample throughput, lower contamination risks, rapid cycling times and high reproducibility have favoured the use of real-time PCR technology in research and surveillance settings (Coertse et al., 2019; Fischer et al., 2013). The target region most often used in the design of real-time PCR primers and probes is the lyssavirus N gene, as it is the most conserved of the lyssavirus genes and allow for the detection of the lyssavirus diversity (Coertse et al., 2019; Marston et al., 2019; Marston et al., 2013; Wunner and Conzelmann, 2013).

2.6.3 Lyssavirus characterisation through full genome determination

The discovery of a novel lyssavirus requires the sequence determination of the full genome regions that include the gene coding regions, UTRs, as well as the genome leader and trailer regions.

Traditionally, genome-walking was used for which primer pairs can be designed according to related reference gene sequences (Marston et al., 2017; Marston et al., 2013; Kuzmin et al., 2008; Tordo et al., 1986). However, disadvantages such as time, effort, related expenses as well as the introduction of PCR errors during Sanger sequencing is problematic when describing novel virus species (Marston et al., 2013; Bracho et al., 1998). The utilizing of Next Generation Sequencing (NGS), a high-throughput sequencing methodology that is used in which millions of sequence reads are generated simultaneously from one sample (Barba and Hadidi, 2017; Marston et al., 2013; Schuster, 2008). The use of NGS also allows for the direct sequencing of large, amplified products, often obtained in amplicon-based technologies, that Sanger sequencing can not accommodate. The implementation of amplicon based NGS does however allow for nucleotide misincorporation and PCR-mediated recombination followed by a degree of incorrect base calling and observed biases during the NGS sequencing (Salk et al., 2018; Goodwin et al., 2016; Robinson and Storey, 2014; Glenn, 2011; Görzer et al., 2010; Taub et al., 2010). This does require sequencing results to be thoroughly inspected and coverage estimates adjusted to allow for non-biased base calling (Taub et al., 2010). However, application of this technology for use in sequencing amplified PCR amplicons to obtain a deep depth of sufficient coverage has been shown

to be highly successful to detect lyssavirus variants which occur in low frequency and are also advantages particularly when detecting and characterising pathogens without prior knowledge of their sequence information (Picard-Meyer et al., 2019; Marston et al., 2017; Barba and Hadidi, 2017; Marston et al., 2014; Nolden et al., 2014; Marston et al., 2013).

2.6.4 Lyssavirus isolation

The isolation of viruses in animal models has been used in laboratories for more than a century (Markotter et al., 2009; Faber et al., 2009). The modern use of cell tissue culture has however caused a decrease in the use of animal models, but in specific instances, there is no substitute. Specific research areas such as evaluation of biologicals, pathogenicity studies and an absence of viable cell tissue culture still require the use of live animal studies. The isolation of specifically lyssaviruses using animal models have shown advantages such as the production of high virus titres without the need for multiple passages, mice being more resistant to contaminants often associated with specimens obtained in the field and high isolation success rate when cell tissue cultures are not available for the specific virus or at the research facility (Markotter et al., 2009; Faber et al., 2009; Bourhy et al., 1989). The usage of animal models does however have disadvantages. The need for trained staff, able to safely and securely handle and inoculate animals is often a limiting factor. The mental effect on staff performing the tests as well as the bioethics and animal welfare implications do warrant the use of cell tissue culture as the preferred method of use, when it is available (Corona et al., 2018).

Chapter 3 – Lyssavirus nucleic acid detection in insectivorous bat species in South Africa

The work reported in Chapter 3 has been accepted for publication in the journal Emerging Infectious Diseases. Modifications
to the manuscript presented in this chapter include more detailed methodology and discussion.
Coertse J, Grobler C, Sabeta C, Seamark E, Kearney T, Paweska J, Markotter W. 2020. Lyssaviruses in insectivorous bats, South Africa, 2003-2018 (In Press)

Introduction

Initially, it was thought that rabies virus (RABV) was the only causative agent of the disease known as rabies. It was only in the late 1950s that rabies-related lyssaviruses were first discovered and described (Shipley et al., 2019; Shope et al., 1970; Boulger and Porterfield, 1958a). The number of described lyssaviruses has more than doubled in the last decade (Kuhn et al., 2020; Markotter and Coertse, 2018). This includes the discovery and identification of several novel lyssavirus species on the African continent. Of the rabies-related lyssaviruses, Lagos bat virus (LBV), Shimoni bat virus (SHIBV), Duvenhage virus (DUVV), Mokola virus (MOKV) and Ikoma lyssavirus (IKOV) are all lyssaviruses that have been found in Africa, whilst virus neutralising antibodies (VNA) for West Caucasian bat virus (WCBV) have been detected in Kenyan bat populations (Markotter et al., 2020; Kuzmin et al., 2010). With the exception of MOKV and IKOV, all the lyssaviruses were identified in various frugivorous and insectivorous bats. LBV has mostly been associated with frugivorous bat species that include Wahlberg's epauletted fruit bat (*Epomophorus wahlbergi*), Egyptian rousette (*Rousettus aegyptiacus*) and Straw-coloured fruit bat (*Eidolon helvum*) while DUVV and SHIBV were found in insectivorous bat species and presumed associated host species being identified as Common slit-faced bat (*Nycteris thebaica*) and striped leaf-nosed bat (*Macronycteris vittatus*) respectively. IKOV and MOKV are not known to be associated with bats to date (Markotter and Coertse, 2018). The only bat lyssavirus from Africa associated with human infections is DUVV which was responsible for the death of three humans in South Africa, Zimbabwe, and the Netherlands (after exposure in Kenya) (Markotter and Coertse, 2018; Meredith et al., 1971; Van Thiel et al, 2009; Paweska et al., 2006). However, the lack of active surveillance programs, the inability of commonly used diagnostic assays (such as FAT) to distinguish between lyssaviruses and limited access to diagnostic facilities have prompted the underestimation of the possible overall public health impact associated with rabies-related lyssaviruses. The association of lyssaviruses from 15 species with insectivorous bats has prompted for the increase in surveillance in specific insectivorous bat species. South Africa hosts a high diversity of insectivorous bats, with multiple bat species such as The Natal long-fingered bat

(*Miniopterus natalensis*), Sundevall's roundleaf bat (*Hipposideros caffer*), multiple *Rhinolophus* spp, *Percival's trident bat* (*Cloeotis percivali*), *N. thebaica* and The Cape hairy bat (*Myotis tricolor*), known to often roost in the same cave systems and have been associated with multiple viral pathogens (Markotter et el., 2020; Kearney et al., 2019; ACR, 2019; Kearney et al., 2017). Three cave systems (Madimatle cave, Matlapitsi cave and Grootboom cave), that are known to harbour multiple insectivorous bats of various species, were selected for routine sampling trips on a monthly basis. Additionally, once off sampling trips to specific locations, located across South Africa, as well as submitted bat samples were also included into the study to provide a representative sample selection. This involved the morphological as well as genetic identification of bat species and the genetic screening for lyssavirus nucleic acids in bat brains. In this study, genetic identification of bat species was performed using a mitochondrial barcoding polymerase chain reaction (PCR) and genetic screening for lyssavirus nucleic acids using a quantitative real-time PCR designed specifically to detect all described lyssavirus RNA at very low copy numbers (Coertse et al., 2019; Coertse et al., 2014; Coertse et al., 2010).

3.1 Materials and methods

3.1.1 Study material

This was a retrospective study consisting of brain samples (n=605) (Appendix A1) of insectivorous bats collected from 2003-2018 across South Africa. Brain samples were collected from bats that were found dead, appeared to be displaying signs of disease or taken as voucher specimens for taxonomic identification in collaboration with the Ditsong National Museum of Natural History (DNMNH) led by Dr Teresa Kearney. Our research group focuses on the study of potential zoonotic pathogens found in bats and small mammals and has well established collaborative efforts with The Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD-NICD), the DNMNH and AfricanBats NPC (Mr Ernest Seamark) that are actively involved in the research of zoonotic pathogens, bat biology and bat conservation in southern Africa. The sampling trips of this study were performed in collaboration with the above-mentioned collaborators along with bat carcasses that have been submitted by bat interest groups and bat rehabilitation centres that include the Gauteng and Northern Regions Bat Interest Group (GNORBIG), ReWild NPC (Jane Burd) as well as suspected possible lyssavirus infected bats submitted to the OIE rabies reference laboratory (ARC-Onderstepoort Veterinary Research Institute).

Routine sampling trips were performed at three sites, the Madimatle cave (24.6181° S, 027.65231° E) in the Limpopo province, Matlapitsi cave (also known as Mahune cave) located near Ga-Mafefe in the Limpopo province (24.114889° S, 30.121158° E) and the Grootboom cave (25.89703° S, 028.22210° E°) in the Gauteng province. The Madimatle cave serves as a maternity roost for *M. natalensis* during certain times of the year but also hosts various bats of other species that include *N. thebaica*, various *Rhinolophus spp*., *H. caffer* as well as *C. percivali*. Grootboom cave is situated in an urban environment and is known as a roost for bats from the *Rhinolophus* and *Miniopterus* genera. The Matlapitsi cave is known to be a roost for bats from several species. These species include the frugivorous bat *R. aegyptiacus* as well as several species of insectivorous bats which include *M. natalensis, H. caffer,* Geoffroy's horseshoe bat (*Rhinolophus clivosus*), Bushveld horseshoe bat (*Rhinolophus simulator*), Blasius's horseshoe bat *(Rhinolophus blasii), C. percivali, N. thebaica* and *M. tricolor*. Samples collected from once off sampling trips as well as vouchers were also included in the study and originated from several locations across South Africa.

3.1.2 Ethical considerations and regulatory requirements

Institutional ethical clearance (ECO54-14) (Appendix B1), Department of Agriculture, Forestry and Fishery (DAFF) (now the Department of Agriculture, Land Reform and Rural Development (DALRRD) section 20 approval (12/11/1/1/8) (Appendix B1) and relevant provincial permits were obtained (Appendix B1). Additional ethical clearance from the Animal (425/2018) and Faculty of Health Sciences Research ethical committee (H007-18) of the University of Pretoria were obtained (Appendix B1).

3.1.3 Biosafety considerations

As the tissue samples could contain different infectious agents and the commercially available rabies vaccine only provides protection against Phylogroup I lyssaviruses, all experimental work with brain tissue (nucleic acid extraction) was performed in an DALRRD approved biosafety level 3 laboratory (Centre for Viral Zoonoses, Faculty of Health Sciences, University of Pretoria) (Appendix B1). In addition to faculty design, safety precautions such as disposable overalls, double gloves, tie-back gowns, eye and face protection and powered, air-purifying respirators (PAPRs) were used.

3.1.4 Sample collection strategies

Samples and data for this study were collected from bats using standard capture and sample collection procedures that have been established by the BEEZ research group in collaboration with Dr. Teresa Kearney (DNMNH), Mr. Ernest Seamark (African Bats NPC) and Prof. Janusz Paweska (Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) at the National Institute for Communicable Diseases (NICD)). Bats were collected in strategically placed two and three bank Austbat harp traps (Faunatech Austbat, Australia) in front of the various cave entrances and mist nets (Ecotone, Poland) were used at various other sampling locations that involved open areas or near known roosts. During sampling trips, captured bats were placed in cotton bags before proceeding to the processing station. Specific on-site morphological identification of insectivorous bat species was conducted by our research team or present collaborators, using body measurements and morphological attributes according to published identification criteria (Kearney and Taylor, 2011; Monadjem et al., 2010; Kearney et al., 2002; Taylor, 2000; Meester, 1986). Voucher specimens were identified and confirmed by Dr. Teresa Kearney (DNMNH) through the use of a morphological identification key (Kearney and Taylor, 2011; Monadjem et al., 2010; Kearney et al., 2002; Taylor, 2000; Meester, 1986). Individuals that were collected as voucher specimens were

anaesthetised using an overdose of Isofor (Safeline pharmaceuticals, South Africa) by inhalation followed by euthanasia by means of cardiac exsanguination (Paweska et al., 2012). Accidental deaths obtained during processing was also subjected to cardiac exsanguination. All tissues such as brain, tongue, lungs, heart, liver, spleen, kidney, intestines, rectum and reproductive organs and wing biopsy were collected during necropsy using sterile instruments and all tissues were collected in individual 1.5 ml microcentrifuge tubes (Sarsted, Germany). These samples are used in our research group to conduct broad pathogen surveillance. This was also performed on the carcasses submitted by the public and the bat interest groups and rehabilitation centres mentioned in Section 3.1.1. The appropriate PPE as described in Section 3.1.3 was used during all sample collection and the necropsy procedures. All samples that were collected in the field were temporarily stored in liquid nitrogen for transportation in accordance with the National Road Traffic Act 93 of 1996 and permanently stored at -80 °C upon return to the BEEZ laboratory in the DALLRD approved Biobank.

3.2.1 RNA extraction

Total RNA was extracted in the BSL-3 laboratory within a biosafety cabinet (Labotec, South Africa). This was done by adding 50-100 mg brain material to 750 µl of Trizol reagent (Invitrogen, United States of America). The sample was then homogenized by vortexing and incubated for 5 minutes at room temperature. Thereafter, 200 µl of chloroform (Merck, Germany) was added, the sample was vigorously shaken and left to incubate at room temperature for 3 minutes followed by centrifugation for 15 minutes at 12 000 g. The aqueous phase was then transferred to a clean 1.5 ml microcentrifuge tube, 500 µl of isopropanol (Merck, Germany) was added, followed by incubation at room temperature for 10 minutes. After incubation, the sample was centrifuged for 30 minutes at 12 000 g. The supernatant was then removed by pipetting, the pellet washed with 75% molecular grade ethanol (Merck, United States of America) and then centrifuged for 5 minutes at 12 000 g. Thereafter the supernatant was removed by pipetting, and the RNA pellet was left to air dry. The RNA pellet was resuspended in 50 µl nuclease-free water (Ambion, United States of America) followed by incubation at 55 °C for 10 minutes. The RNA was stored at -20 °C for short term storage during usage of RNA followed by long term storage at -80 °C.

3.2.2 Quantitative real-time reverse transcription polymerase chain reaction

A quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) that detects lyssavirus from all species even at low copy numbers was used (Coertse et al., 2019; Coertse et

al., 2014; Coertse et al., 2010). The specific qRT-PCR uses the Takara One Step PrimeScript™ RT-PCR kit (Takara Bio Inc, Japan) and amplifies a 126 bp region of the conserved nucleoprotein gene and indicates amplification by fluorescence using a 5'-nuclease fluorescent probe (Coertse et al., 2019). A final volume of 9 µl containing 2.4 µl nuclease free water (Ambion, United States of America), 5 µl One step RT-PCR Buffer III (2X) (Takara Bio Inc, Japan), 0.2 µl TaKaRa Ex Taq HS (5U/µl)(Takara Bio Inc, Japan), 0.2 µl PrimeScript RT Enzyme Mix II (Takara Bio Inc, Japan), 0.4 µl 541lys (20 pmol)(Table 3.1), 0.4 µl 550B primer (20 pmol)(Table 3.1), 0.4 µl 620lyssaC probe (10pmol)(Table 3.1) was added to a 1.5 ml microcentrifuge tube to prepare the master mix. The reagents were briefly centrifuged, and 9 µl of the master mix was transferred to a 0.2 µl optical PCR tube (Life technologies, China) followed by 1 µl of the sample RNA. Thermocycling conditions for the qRT-PCR were 1 cycle of reverse transcription with 1 step of 42 °C for 30 minutes and 1 step of 95 °C for 10 seconds. Then PCR for quantification for 45 cycles of 95 °C for five seconds, 42 °C for five seconds (single acquisition mode) and 72 °C for five seconds. The QuantStudio 5 Real-Time PCR System (Applied Biosystems) was used, and analysis of results was done using the Quantstudio design and analytic software version 1.3.1. Quantification was performed using an external standard curve previously described (Coertse et al., 2010, Coertse et al., 2014). *In vitro* transcribed RNA of the target region (RABV, CVS-11, Genbank accession number M13215) at a concentration of 10^4 copies/µl was used as a positive control, and a no-template control was also included. All samples producing a positive result, were subjected to a conventional RT-PCR or hemi-nested PCR to increase the size of the amplified region to perform DNA sequencing and subsequent phylogenetic analyses.

Table 3.1 Oligonucleotide primers for cDNA synthesis, quantitative real-time RT-PCR, conventional RT-PCR amplification and hemi-nested PCR amplification of lyssaviruses (Coertse et al., 2019)

Primer	Sequence 5'-3'	Application	Genome binding position	Reference
001lys	ACG CTT AAC GAM AAA	cDNA synthesis, PCR	1-15	(Markotter et al., 2006)
550B	GTR CTC CAR TTA GCR CAC AT	real time RT-PCR, PCR	647-666	(Markotter et al., 2006)
304	TTG ACA AAG ATC TTG CTC AT	PCR	1514-1533	(Markotter et al., 2006)
541lysfor	CAC MGS NAA YTA YAA RAC NAA	real time RT-PCR	541-561	(Coertse et al., 2010)
JW12	ATG TAA CAC CYC TAC AAT G	PCR	55-74	(Heaton et al., 1997)
620lyssaC probe	(FAM)-CAY CAY ACH YTV ATG ACH ACH CAY AA-(QSY)	real time RT-PCR	620-645	(Coertse et al., 2019)

Degenerate bases- **R**- A/G, **S**- G/C, **M**- A/C, **Y**- C/T, **H**- A/C/T, **V**- A/C/G, **N**- any base
FAM- 6-carboxyfluorescein, QSY- non-fluorescent quencher
Genome binding positions are numbered according to the Pasteur virus sequence (GenBank accession number M13215)

3.2.3 Conventional RT-PCR amplification of lyssavirus full N gene

A conventional RT-PCR was performed on all samples that produce a positive result during the qRT-PCR. The conventional RT-PCR was performed to confirm qRT-PCR results and allow for the amplification of the full lyssavirus N-gene to perform gene sequencing and phylogenetic analysis. For the conventional RT-PCR, reverse transcription was performed on the extracted RNA as described in Section 3.2.1, by adding 1 µl of 001lys primer (10 pmol) (Table 3.1), 1 µl dNTP Mix (10 mM) (Thermo Fisher Scientific, United States of America), 5 µl of RNA and 6.6 µl of nuclease-free water (Ambion, United States of America) to a 0.2 ml thin-walled PCR tube. *In vitro* transcribed RNA (RABV, CVS-11, Genbank accession number M13215) of the full N gene was used as a positive control, and a no-template control was included. The contents were briefly centrifuged and then incubated at 65 °C in for 5 minutes. The tube was placed on ice for 1 minute. A master mix containing 4 µl First-strand Buffer (5X) (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$) (Thermo Fisher Scientific, United States of America) , 1 µl of Dithiothreitol (DTT) (0.1 M) (Thermo Fisher Scientific, United States of America) , 1 µl Ribolock RNase inhibitor (40 U/µl) (Thermo Fisher Scientific, United States of America), and 1 µl SuperScript™ III RT enzyme (200 U/µl) (Thermo Fisher Scientific, United States of America) was added to each tube and incubated at 55 °C for 60 minutes followed by inactivation at 70 °C for 15 minutes using a dry heating block (Labnet International, Inc, United States of America). The cDNA was then stored at -20 °C.

The conventional PCR used for the amplification of the full N-gene of lyssaviruses uses the 001lys primer (Table 3.1) and the 304 primer (Table 3.1) to amplify a 1353 bp region of the well-conserved nucleoprotein gene. The gene was amplified in a final volume of 100 µl containing 66.5 µl nuclease free water (Ambion, United States of America), 10 µl Dream Taq PCR Buffer (10x) (containing KCl, $(NH_4)_2SO_4$ and 20 mM of MgCl2)(Thermo Fisher Scientific, United States of America), 1 µl 001lys primer (10 pmol)(Table 3.1), 1.25 µl 304 primer (10 pmol)(Table 3.1), 1 µl dNTP Mix (10mM) (Thermo Fisher Scientific, United States of America) and 0.25 µl Dream Taq Polymerase (5U/µl) (Thermo Fisher Scientific, United States of America) and 20 µl of the sample cDNA (prepared). *In vitro* transcribed RNA (RABV, CVS-11, Genbank accession number M13215) of the full N gene was used as a positive control, and a no-template control was included. Amplification was performed on an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, United States of America) under the following conditions: 94 °C for 1 minute, 40 cycles of 94 °C for 30 seconds, 37 °C for 30 seconds, 72 °C for 2 minutes, followed by final extension at 72 °C for 7 minutes and held at 4 °C.

3.2.4 Visualisation of amplified PCR products

Amplified products were visualised by agarose gel electrophoresis (1.5 % (w/v) agarose, 120V for 40 minutes) in 1 x Tris-acetate EDTA (TAE) electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA) (Thermo Fisher Scientific, United States of America), 1 µg/ml ethidium bromide (Life Science, VWR, United States of America) and UV illumination with a 100 bp molecular marker for size reference (Thermo Fisher Scientific, United States of America). If the correct band sizes were noted, they were excised from the gel and purified using the Zymoclean™ Gel DNA recovery kit (Zymo Research Corp, United States of America). This was done by adding three volumes of agarose dissolving buffer (ADB) (Zymo Research, United States of America) to each volume of agarose excised from the gel. The tube was then incubated at 55 °C until the gel slice was completely dissolved. The melted agarose solution was then transferred to a Zymo-Spin column in a collection tube and then centrifuged for 60 seconds at 16 000 *g*. Thereafter 200 µl of DNA wash buffer (Zymo Research) was added to the column followed by centrifugation at 16 000 *g*. The flow-through was discarded, and the wash step repeated, followed by the addition of 40 µl DNA elution buffer (Zymo Research) directly to the column matrix and incubation at room temperature for 1 minute. The solution was then centrifuged for one minute at 16 000 *g* and stored at -20 °C.

3.2.5 Sequencing of amplified products

A 10 µl sequencing PCR reaction was prepared in a 0.2ml PCR tube containing the following: 1 µl of either the reverse or forward primer (3.2pmol) (Table 3.1), 2.1 µl of sequencing buffer (5X) (Thermo Fisher Scientific, United States of America), 0.5 µl of the BigDye® Terminator v3.1 (Thermo Fisher Scientific, United States of America). The volume of DNA product (1-10 µl) to be added to the reaction was determined running 1µl of the purified DNA and 1µl GeneRuler 100bp DNA Ladder (0.5µg/µl) (Thermo Fisher Scientific, United States of America) on an agarose gel electrophoresis as previously described. The concentration of the band was compared against the concentrations of marker bands in the GeneRuler 100bp DNA Ladder (0.5µg/µl) (Thermo Fisher Scientific). The reactions were briefly centrifuged at 500 g for 3 seconds and incubated in an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, United States of America) at 96 °C for 60 seconds; 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes; and held at 4 °C. The sequencing reaction products were then purified using the Sodium Acetate/Ethanol precipitation method by mixing 10 µl PCR reaction with 10 µl of nuclease-free water (Ambion, United States of America) to a final volume of 20 µl and transferred into a 0.5 ml microcentrifuge tube. To each reaction 62.5 µl of 100% molecular grade ethanol (Merck, Germany), 3 µl Sodium Acetate (NaOAc) (3M, pH 4.6) (Thermo Fisher Scientific, United States of America) and 14.5 µl nuclease-free water (Ambion, United States of America) was added and centrifuged at 12 000 *g* for 30 minutes. The supernatant was discarded by careful removal of the aqueous phase. The pellet was immediately washed by adding 250 µl of cold freshly prepared 70% ethanol (Merck, Germany) and was centrifuged at 12 000 *g* for 5 minutes. The supernatant was carefully removed. The wash step was performed twice to remove all other impurities. The purified pellet was submitted to the sanger sequencing facility at the University of Pretoria, Faculty of Natural and Agricultural Sciences for sequencing, on an automated ABI3500xl genetic analyzer (Thermo Fisher Scientific, United States of America).

3.2.6 Phylogenetic analyses of full N gene

The raw sequences generated were analyzed with BioEdit Alignment Editor Version 7 (Hall, 1999). Manual base-calling was done where needed and nucleotide sequences were trimmed. The edited forward and reverse sequences were aligned using the Clustal W multiple alignment function of the BioEdit software to obtain a consensus sequence. The obtained consensus sequences were then compared to all other known lyssavirus sequences using the Clustal W

multiple alignment function in the BioEdit software. Phylogenetic relationship comparison of the sequences was performed using Bayesian inference (BEAST package) to determine relatedness of the viral sequences to other known lyssaviruses. The aligned sequences were analysed using the JModel test software v.2.1.7 to find the best-fit DNA substitution model using the Bayesian Information Criterion (BIC) (Darriba et al., 2012). Bayesian Evolutionary Analysis Sampling trees (BEAST) software v1.8.4 with the Bayesian MCMC chains set at 10 000 000 repeats sampling every 1 000 trees, general time-reversible substitution model as determined by jmodeltest2, invariant sites, and gamma distribution was used. We assumed an underlying coalescent process with constant population size (Drummond et al., 2012). Trees were generated using Tree annotator (v10.1.4) with a burn in value of 10%. Thereafter viewing and editing of trees was performed using Figtree v1.4.0. For further phylogenetic analyses, MEGA X (Kumar et al., 2018) was also used employing the maximum likelihood method with a 1000 bootstrap replicates using a general time-reversible substitution model as determined by jmodeltest2 in MEGA X. Pairwise similarities between viral sequences were determined and analysed in MEGA X with pairwise deletions.

3.2.7 Bat species identification using DNA barcoding

DNA sequences were used to confirm morphological identification of bat species based on genetic differences. All lyssavirus qRT-PCR positive samples as well as samples where the morphological identity of the bat species were uncertain, were subjected to genetic identification.

3.2.7.1 DNA extraction

DNA was extracted from wing biopsy (heart or pectoral muscle if wing biopsy was not available) using the Quick-DNA™ Miniprep Kit (Zymo Research, United States of America). Wing biopsy tissue (or other tissue) was mechanically homogenized (up to 25 mg of fresh or frozen tissue) in 500 µl of genomic Lysis Buffer (Zymo Research, United States of America). The lysate was then centrifuged at 10,000 g for 5 minutes. Making sure not to disturb the pelleted debris, the supernatant was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000 g for one minute. Thereafter the flow through was discarded. The Zymo-Spin™ IIC column (Zymo Research, United States of America) was transferred to a new collection tube followed by the addition of 200 µl of DNA pre-wash buffer (Zymo Research, United States of America) to the spin column. The column was then centrifuged at 10,000 g for one minute. Then 500 µl of genomic-DNA wash buffer (Zymo Research, United States of America) was added to the spin column followed by centrifugation at 10,000 g for one minute. The spin column was transferred

to a clean microcentrifuge tube and 50 µl DNA elution buffer (Zymo Research, United States of America) was added to the spin column. The column was incubated for 5 minutes at room temperature and then centrifuged at 16 000 g for 30 seconds to elute the DNA. The eluted DNA was stored at -20℃.

3.2.7.2 Barcoding Polymerase chain reaction (PCR)

A barcoding PCR was performed to amplify a partial fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI). The selected primers bind and amplify a region of 708 bases (gene region reference: 5369- 6077 (*Rhinolophus thomasi*, Genbank accession KY124333.1). For each reaction, a total volume of 50 µl PCR reaction mixture was prepared. Each reaction contained: 2 µl of forward and reverse primer (10 pmol)(Table 3.2), 1 µl dNTPs (10mM) (Thermo Fisher Scientific, United States of America), 0.25 µl Taq polymerase (5U/µl) (Thermo Fisher Scientific, United States of America), 1.5 µl $MgCl_2$ (25mM) (Merk, Germany), 5 µl Dream Taq buffer (10X) (containing KCl, $(NH_4)_2SO_4$ and 20 mM of $MgCl_2$) (Thermo Fisher Scientific, United States of America), 5 µl sample DNA template and 33.25 µl nuclease free water (Ambion, United States of America). Amplification was performed on an Applied Biosystems SimpliAmp Thermal Cycler with the following temperature cycling; initial single denaturation step at 94 ℃ for 5 minutes, followed by 45 cycles of 94 ℃ for 2 seconds, 94 ℃ for 30 seconds, 48 ℃ for 50 seconds, 72 ℃ for 1 minute, 72 ℃ for 10 minutes and then hold on 4 ℃.

Table 3.2 Oligonucleotide primers used for DNA barcoding PCR of Mitochondrial cytochrome C oxidase subunit one gene (CO1) gene region amplification.

Gene target	Primer sequence 5'-3'	Primer binding position on reference genome	Reference
Mitochondrial cytochrome C oxidase subunit one gene (CO1)	Folmer LCO1490 For- GGT CAA CAA ATC ATA AAG ATA TTG G	Position 5369-5394	(Folmer, 1994)
Mitochondrial cytochrome C oxidase subunit one gene (CO1)	Folmer HCO2198 Rev- TAA ACT TCA GGG TGA CCA AAA AAT CA	Position 6051-6077	(Folmer, 1994)

* Reference genome- *Rhinolophus thomasi* (Genbank accession KY124333.1)

3.2.7.3 Barcoding amplified product sequencing and phylogenetic analysis

The amplified products were visualised on agarose gels post-electrophoresis, purified using the Zymoclean^{TM} Gel DNA recovery kit (Zymo Research, United States of America), subjected to a sequencing reaction and submitted to the Sanger sequencing facility at the University of Pretoria, Faculty of Natural and Agricultural Sciences for sequencing, as described in Section 3.1.3 and 3.2.5. Obtained sequences were manually edited using BioEdit Version 7 (Hall, 1999) and compared to available reference nucleotide sequences identified in GenBank using the BLASTn function as well as the Barcode of Life Data (BOLD) system 3 (Ratnasingham and Hebert, 2007). Obtained nucleotide sequences were compared to the bat barcode sequences identified on GenBank and BOLD using pair-wise nucleotide similarities to confirm bat identifications. These genetic classification results were also compared to similar species previously identified in our research group's bat species database as well as with previously morphologically identified voucher specimens performed by Dr. Teresa Kearney (DNMNH).

3.3 Results

3.3.1 Quantitative real-time reverse transcription PCR screening

To determine if lyssavirus RNA was present in the insectivorous bat populations in South Africa, a total of 605 individual brain samples were tested using the quantitative real-time reverse transcription PCR as described in Section 3.2.2. The sample set tested represented a total of 5 bat families and 41 different species tested from across South Africa (Table 3.3; Figure 3.1).

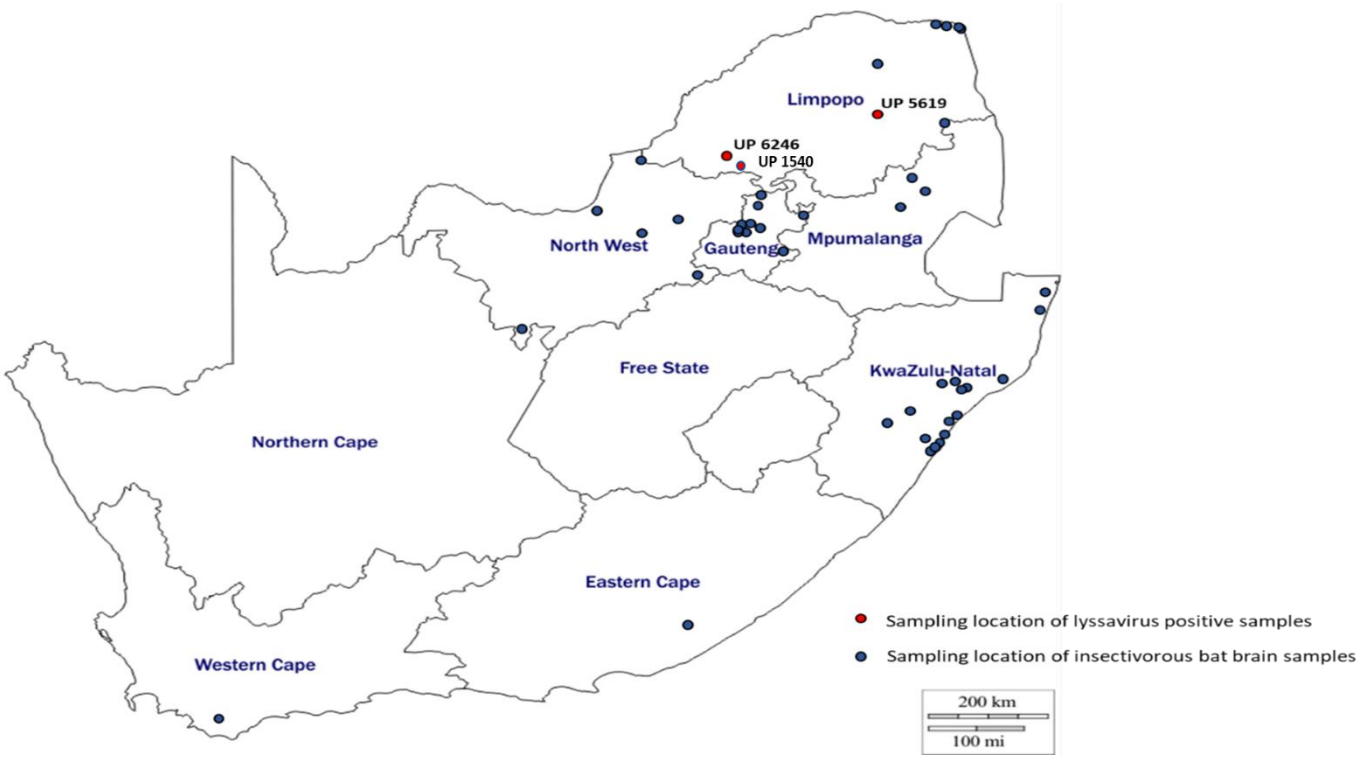


Figure 3.1 Map of South Africa indicating the locations where insectivorous bat samples tested in this study were collected (blue) and sampling location of bats that tested positive for lyssaviruses (red).

Table 3.3 Number of individuals of different bat species, collected from South African provinces during 2003-2018 for lyssavirus surveillance.

Family	Species	EC	GP	KZN	LP	MP	NW	Total
Molossidae	*Chaerephon (Mops) ansorgei*				2			2
	Chaerephon (Mops) pumilus			15	3			18
	Mops condylurus		1		8			9
	Mops midas				3			3
	Otomops martiensseni			7				7
	Sauromys petrophilus						1	1
	Tadarida aegyptiaca		10	2			5	17
Hipposideridae	*Cloeotis percivali*				5			5
	Hipposideros caffer				16	1		17
Nycteridae	*Nycteris thebaica*		14	9	18		1	42
	Eptesicus hottentotus						1	1
Vespertilionidae	*Glauconycteris variegata*			3	1			4
	Kerivoula (Glauconycteris) argentata			1				1
	Laephotis botswanae		1	1				2
	Miniopterus fraterculus		1		2		2	5
	Miniopterus natalensis		30	8	80		6	124
	Myotis tricolor				9	2		11
	Myotis welwitschii					1		1
	Neoromicia (Laephotis) capensis		42	3	7	4	11	67
	Neoromicia (Laephotis) cf helios				4			4
	Neoromicia (Laephotis) helios				2			2
	Neoromicia (Laephotis) nana			2	8	3		13
	Neoromicia (Laephotis) rendalli				1			1
	Neoromicia (Laephotis) zuluensis				2	1		3
	Nycticeinops schlieffeni				8			8
	Pipistrellus hesperidus	7	1	9	2	1		20
	Pipistrellus rusticus		23		8	1	3	35
	Scotophilus dinganii		5	14	18		12	49
	Scotophilus leucogaster				5			5
	Scotophilus viridis				7			7
Rhinolophidae	*Rhinolophus blasii*				12			12
	Rhinolophus clivosus		2	10	17	1		30
	Rhinolophus damarensis						5	5
	Rhinolophus darlingi			1	4			5
	Rhinolophus denti						5	5

	Rhinolophus hildebrandtii s.I.				4			3
	Rhinolophus landeri				1			1
	Rhinolophus simulator				48		2	50
	Rhinolophus smithersi				4			4
	Rhinolophus swinnyi			3				3
Emballonuridae	*Taphozous mauritianus*				3			3
Total		**7**	**130**	**88**	**309**	**17**	**54**	**605**

Province abbreviations used in table: Eastern Cape (EC), Gauteng (GP), KwaZulu-Natal (KZN), Limpopo (LP), Mpumalanga (MP), North West (NW)
Updated taxonomic names are indicated in parentheses

The samples were collected from five different provinces in with the majority of samples originating from the Limpopo province (Table 3.3). A large proportion of bats collected in the study did not display any signs of disease (n=562 (92.9%)). -Sentence changed from "A total of 28 (4.7%) bats were collected dead at the site where sampling was conducted, 12 (1.9%) bats displayed signs of disease or abnormal behaviour that included being grounded, appeared disoriented, partial or full paralysis, or displayed lethargic behaviour." to "A total of 28 (4.7%) bats were collected dead at the site where sampling was conducted, 12 (1.9%) bats displayed signs of disease or abnormal behaviour that included being grounded, appeared disoriented, partially or fully paralysed. From the 605 insectivorous bat brain samples tested, only three samples, UP 1540, UP 5619 and UP 6246 (Table 3.4), yielded a positive real time PCR result. Ct values of 29.9 (UP 5619) and 19.38 (UP 6246) and RNA copy numbers of 2601 (UP 5619) and 1076 655 (UP 6246) (Figure 3.2) were obtained for two of the positives samples. UP 1540 was first detected in a previous study but further sequence analyses and characterisation was not performed until this study (McCulloch, 2013). Unfortunately, the initial real time PCR Ct and copy number result of UP 1540 is not available.

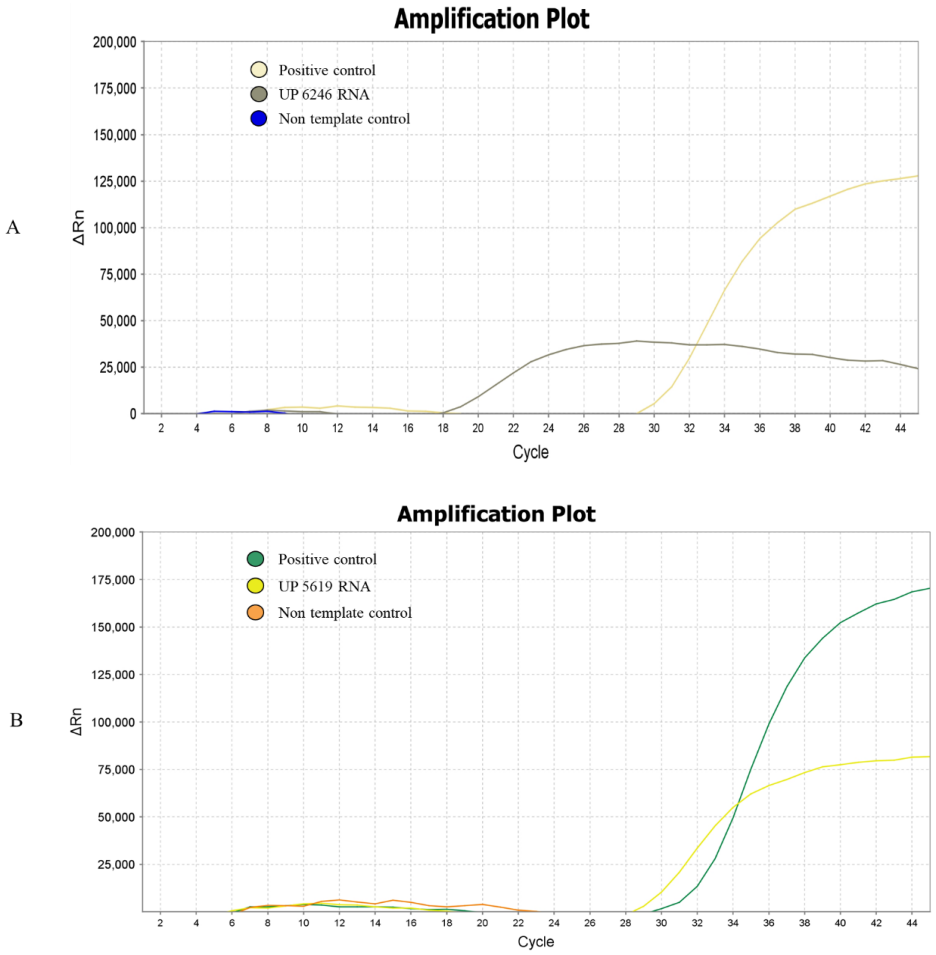


Figure 3.2 A-Amplification curve of the real-time PCR targeting the 126 bp partial nucleoprotein gene showing amplification results of UP 6246 identified during this study. B-Amplification curve of the real-time polymerase chain reaction targeting the 126 bp partial nucleoprotein gene showing amplification results of UP 5619 identified during this study.

Table 3.4 Summary of information pertaining to the threelyssavirus nucleic acid positive bats detected in this study.

Sample number	Confirmation of species identification	Site	Locality	Province	Country	Dec (S)	Dec (E)	SEX
UP 1540	*Nycteris thebaica*	Rooiberg	Rooiberg, Thabazimbi	Limpopo	South Africa	24.77749	27.73408	F
UP 5619	*Miniopterus natalensis*	Matlapitsi cave	Ga-Mafefe	Limpopo	South Africa	24,11487	30,12151	M
UP 6246	*Miniopterus natalensis*	Madimatle Cave	Meletse, Thabazimbi	Limpopo	South Africa	24,6181	27,65231	F

3.3.2 Phylogenetic analyses of lyssavirus full N gene

Bayesian phylogenies as well as maximum likelihood analysis were constructed using the amplified full N gene sequences (Drummond et al., 2012; Kumar et al., 2018). To determine the phylogeny position of the three identified lyssavirus positive samples, they were compared to the described lyssavirus diversity (Figure 3.3; Figure 3.4).

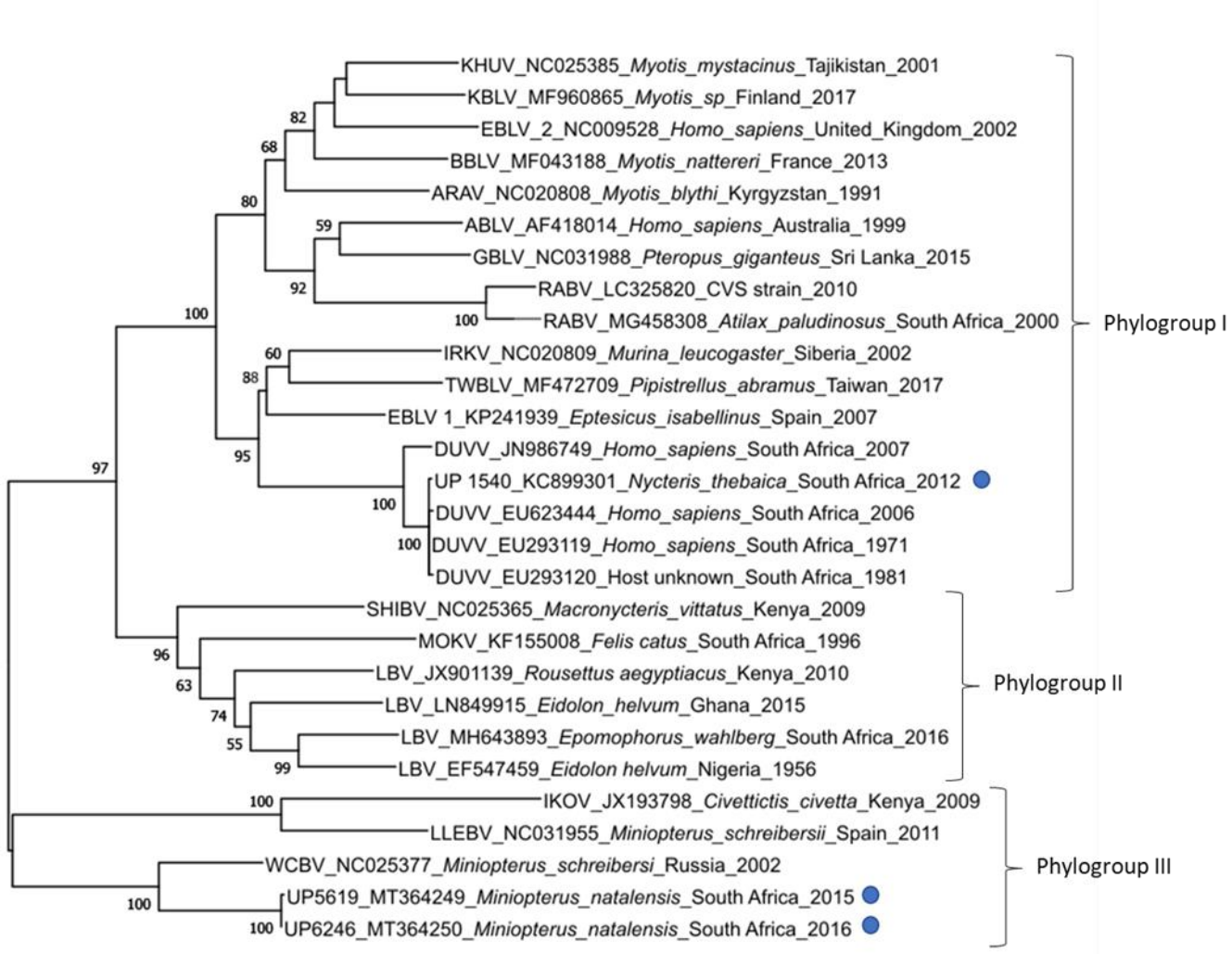


Figure 3.3 Maximum likelihood analysis of the relatedness of the lyssavirus full N gene of three positive samples generated in this study, constructed using MEGA X using the GTR+G+I model. The analysis is based on a 1356 bp fragment of the lyssavirus N gene sequences with a bootstrap of 1000 replicates. Only bootstrap values above 50 are shown. Lyssavirus positive samples detected in this study are marked with blue dots

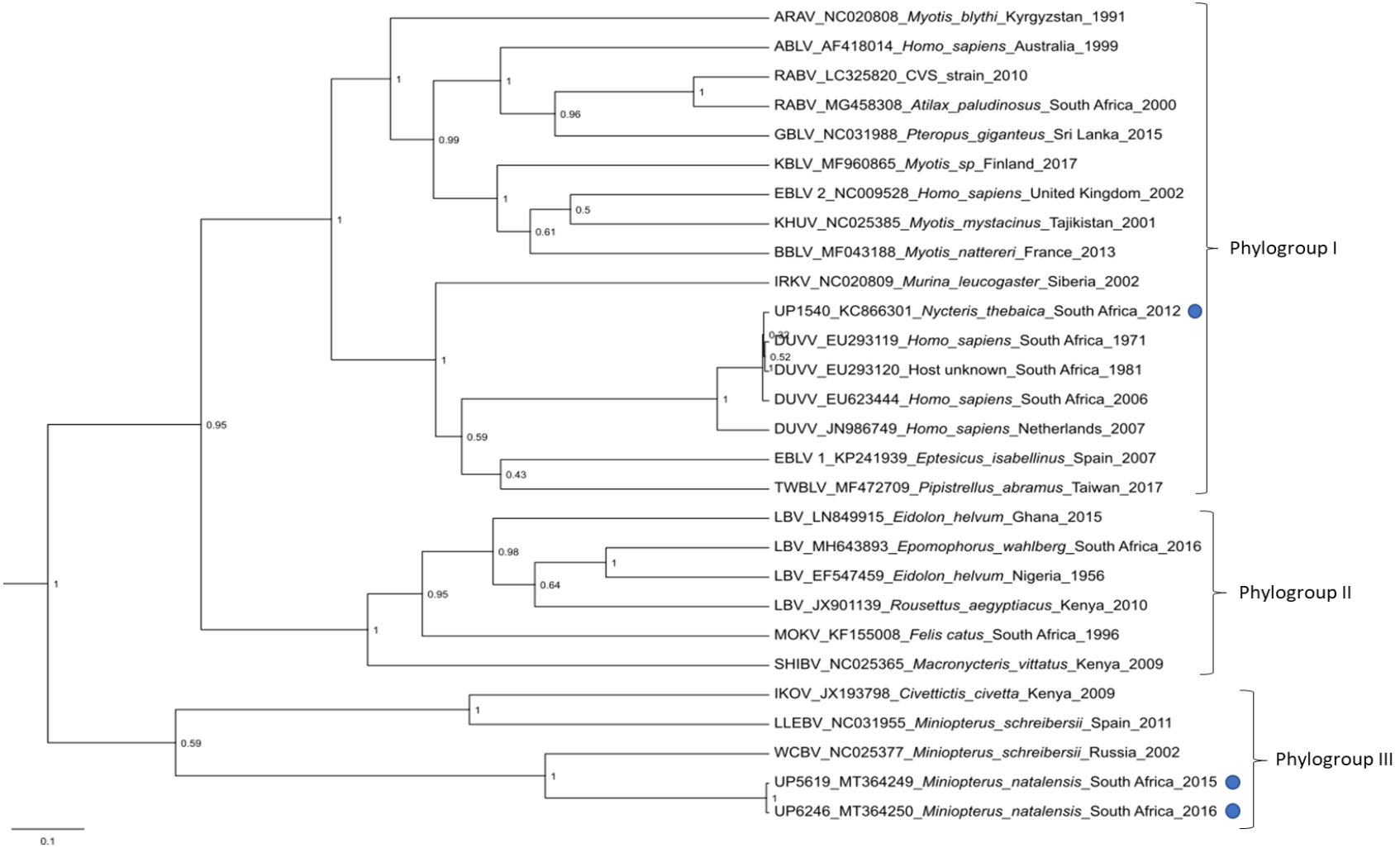


Figure 3.4 Phylogenetic analyses of three lyssavirus positive samples full N gene sequences generated in this study. Bayesian phylogeny of approximately 1356 bp of the conserved full N gene was achieved using BEAST employing the GTR+I+G substitution model for posterior probabilities at MCMC chain setting of 10M samples every 1000 states. Lyssavirus positive samples detected in this study are marked with blue dots.

Pairwise distance analyses were performed comparing the full N gene sequences of the newly identified and other lyssaviruses. Full N gene sequence obtained from UP 1540 grouped within the DUVV clade and showed a sequence identity ranging between 91.6-99.3% when compared to previously described DUVVs (Table 3.6). Both full N gene sequences obtained from UP 5619 and UP 6246 grouped closest to WCBV with 80.9-81% sequence identity (Table 3.5). When comparing pairwise distances between the obtained UP 5619 and UP6246 N gene sequences, there was an estimated 99.2% sequence identity.

Table 3.5 Nucleotide identity between different full nucleoprotein gene sequences obtained from UP 5619, UP 6246 and phylogroup III lyssaviruses. There was a total of 1356 positions in the final dataset. Evolutionary analyses were conducted in MEGA X using the Maximum Composite likelihood model.

	1	**2**	**3**	**4**
1- UP 5619 MT364249 *Miniopterus natalensis* South Africa 2015				
2- UP 6246 MT364250 *Miniopterus natalensis* South Africa 2016	0,99			
3- WCBV NC025377 *Miniopterus schreibersi* Russia 2002	0,81	0,81		
4- IKOV JX193798 *Civettictis civetta* Kenya 2009	0,70	0,71	0,72	
5- LLEBV NC031955 Spain 2011	0,71	0,71	0,72	0,75

Table 3.6 Nucleotide identity between different full nucleoprotein gene sequences obtained from UP 1540 and previous DUVV isolates. There was a total of 1356 positions in the final dataset. Evolutionary analyses were conducted in MEGA X using the Maximum Composite likelihood model.

	1	**2**	**3**	**4**
1- UP1540 KC866301 *Nycteris thebaica* South Africa 2012				
2- DUVV EU293119 *Homo sapiens* South Africa 1971	0,99			
3- DUVV EU623444 *Homo sapien* South Africa 2006	0,99	0,99		
4- DUVV EU293120 Host unknown South Africa 1981	0,99	0,99	0,99	
5- DUVV JN986749 *Homo sapien* Netherlands 2007	0,92	0,92	0,91	0,92

3.3.3 Bat species identification using DNA barcoding

UP 1540 was morphologically identified as a *N. thebaica*. *N. thebaica* are morphologically distinct from any known related bat species found in South Africa, thus DNA barcoding was not a necessity for accurate species identification (Monadjem et al., 2009). Nucleic acid identity obtained from UP 5619 and UP 6246 bat samples were analysed and compared to available bat species COI sequences available on GenBank as well as BOLD (Table 3.7). Both COI sequences for UP 5619 and UP 6246 showed a high sequence similarity of between 98.85- 98.87% and 98.06- 99.04 %

respectively, to sequences publically available. The closest match to the COI sequences for UP 5619 and UP 6246 was *M. natalensis* (Genbank: KF452625, BOLD: ADX6002). This specific sequence was obtained from a male bat, during 2007 from the North West province of South Africa. The bat was morphologically identified by Dr. Teresa Kearney (DNMNH) and the voucher specimen is available in their collection (museum number: TM48022). Both UP 5619 and UP 6246 were subsequently identified as *M. natalensis*.

Table 3.7 Summary of the BLASTn and BOLD taxanomic search results obtained for the molecular identification of two lyssavirus positive bat species.

	GenBank			**BOLD**		
	Percentage identity	**Accession number**	**Confirmed matched species**	**Percentage identity**	**Bin ID**	**Confirmed matched species**
UP 5619	98.87 %	KF452625*	*Miniopterus natalensis*	98.85 %	BOLD: ADX6002 (KF452625)*	*Miniopterus natalensis*
UP 6246	98.06 %	KF452625*	*Miniopterus natalensis*	99.04 %	BOLD: ADX6002 (KF452625)*	*Miniopterus natalensis*

* KF452625- *Miniopterus natalensis*, museum number - TM48022 (Dr T. Kearney), Madikwe Game reserve, North West province, South Africa

3.4 Discussion

A total of 605 brain samples were tested representing 5 different bat families and a total of 41 different insectivorous bat species. It was found that only 12 bats (1.9 %) showed signs of any abnormal behaviour potentially associated with disease. This is often seen, as bats are known to tolerate pathogen infection due to abortive infection taking place as discussed in Section 2.4. The sample set used in this study did allow for a geographical bias towards the Limpopo province due to the routine sampling trips that were performed at the caves within this province. This, along with the opportunistic nature of sample collection and submissions from across South Africa, allowed for the variance in the number of each specific bat species represented in the sample set. Three samples tested positive for the presence of lyssavirus nucleic acids. One positive sample (UP 1540) was obtained from a *N. thebaica* and was identified to be DUVV based on nucleotide similarity and strong Bayesian and maximum likelihood tree topology. The sample was collected in August 2012 about six kilometres outside the town of Rooiberg, Limpopo province, during a survey undertaken by members of a bat interest group (GNoR BIG). During initial sampling by the bat interest group, it was noted that the specific *N. thebaica* was significantly smaller than the other captured *N. thebaica*s and was easily caught with a hand net. The following morning that individual bat was found dead in the place where it was released during the previous night. The carcass was then sent to the University of Pretoria for pathogen testing. This DUVV infection is now the second documented in *N. thebaica* bats. Therefore, *N. thebaica* appears to be the only bat species identified as DUVV host to date. *N. thebaica* is widely distributed throughout Africa and is known to often co-roost with bats of various other species. The other two lyssavirus positive samples (UP 5610 and UP 6246) were obtained from *M. natalensis* that were described as apparently healthy individuals that did not display any signs of illness and did not display any notable abnormal behaviour. Morphological characteristics in addition to molecular identification confirmed that both bats were *M. natalensis*. These bats are known to be distributed throughout South Africa and are primarily found roosting in cave systems or mines (Pretorius et al., 2020; Hes and Mills, 1997). *M. natalensis* is classified as a migratory insectivorous bat species in South Africa, known to often travel large distances, crossing provincial bounders during seasonal migration between winter and autumn hibernacula roosts and the summer maternity cave roosts, forming maternal colonies where pups are be born (Miller-Butterworth et al., 2005; Hes and Mills, 1997). They are also known to co-roost with bats of several other species including *C. percivali, N. capensis, H. caffer, M. tricolor* and several *Rhinolophus* spp. The bat UP 5619 was sampled at the Matlapitsi cave located in Gamafefe while the bat UP 6246 was sampled at the Madimatle cave in Meletse near Thabazimbi (Table 3.4), both in the Limpopo province with a distance of 252 km

from each other. Both these caves are known to host multiple insectivorous bat species, additionally, the Matlapitsi cave also harbours *R. aegyptiacus*, a frugivorous bat, known to be the host for LBV. The two caves from which the positive bats were sampled are also known to be used for traditional, recreational and religious purposes meaning frequent entering of humans into the caves without any personal protective clothing or equipment. The Matlapitsi cave is also situated within a rural area where local agricultural practices for vegetable, fruit and livestock farming. Domestic animals (mainly dogs) are also free roaming in the area. As *M. natalensis* movement patterns and the interaction with different bats inside caves are not well documented, the exact geographical and possible host range cannot be evaluated. Considering that bats of certain species are known to undertake regional migrations for several hundred kilometres, the possibility of virus spread and transmission across and between bat roosts poses a threat of these viruses being present in other bat roosts across South Africa (Rodrigues and Palmeirim, 2008). The frequent entering of humans into the caves as well as the setting of the caves in rural areas also provides opportunities for spillover events in humans as well as in livestock and domestic animals.

The three samples tested positive for the presence of lyssavirus nucleic acids representing a prevalence of 0.5 % out of all samples screened in this study. Similar prevalence rates have been seen in other lyssavirus surveillance studies (Markotter and Coertse, 2018; Rupprecht et al., 2011). Initial sequence data obtained from the full N gene showed a mere 2.2 % nucleotide difference between the *M. natalensis* isolates, suggesting that both these viruses belong to the same viral species. However, the next closest related virus, WCBV, showed a 19 % nucleotide difference when comparing the N gene. As explained in Section 2.1, two of the International Committee on Taxonomy of Viruses' (ICTV) criteria for lyssavirus species classification is the genetic distance of the complete N gene with a threshold of between 80-82% nucleotide identity as well as a need for consistency in phylogenetic trees that have been obtained using various evolutionary models. A more than 18 % nucleotide divergence obtained in the full N gene comparison as well as consistent and clear topological grouping using bayesian inference and maximum likelihood phylogenetic models, is indicatory of a potential new lyssavirus species and determination of the complete genome is necessary (See Chapter 4). The grouping of the two novel lyssaviruses out of Phylogroup I suggests that the conventional RABV-based vaccines will not provide a degree of protection/cross neutralisation and thus conventional post exposure prophalaxis will not be successful against these pathogens (Horton et al., 2014; Weyer et al., 2008; Hanlon et al., 2005; Nel et al., 2003). Further serological cross-reactivity studies can potentially provide informative results on the protective/cross neutralisation possibly offered by the conventional RABV vaccine.

Whether the insectivorous bat, *M. natalensis*, is the actual host or perhaps a spillover host is not known and more research into the definitive host is required. Both caves where UP 5619 and UP 6246 were collected harbour bats of multiple species throughout the year and the possibility of a spillover from a bat from another species cannot be excluded. Additional serological surveillance is necessary to confidently determine host range and distribution patterns. Such serological surveillance is cumbersome as closely related lyssaviruses will exhibit certain degree of cross-neutralisation. Serological studies will have to be interpreted with cautionHowever, the identification of lyssavirus nucleic acids, sampled from different locations in Limpopo, does provide evidence of the presence of known and novel lyssavirus species in insectivorous bat populations. The analyses of full N gene indicated this new detection to be a potential new lyssavirus species, preliminary named Matlo bat lyssavirus (MBLV) after the province and cave site where the positive bat was sampled.

Geographically, MBLV was isolated in *M. natalensis* in the northern regions of South Africa, whereas the most closely related lyssavirus was isolated from a different bat species, M. schreibersii, in Eurasia. The geographical distance as well as different host species between isolations provides further evidence for the classification of MBLV as a separate and distinct lyssavirus species.

Chapter 4 – Virus isolation and full genome characterisation of Matlo bat lyssavirus

Introduction

The increase in the number of identified novel viruses can be seen in the recent diversification of the genus *Lyssavirus*, family *Rhabdoviridae*, with the number of described lyssavirus species doubling in the past decade (Markotter and Coertse; 2018; Horton et al., 2014). The genus currently consists of 17 formally recognised viral species with one tentative species awaiting classification from the International Committee on Taxonomy of Viruses (ICTV). The criteria used during the classification of possible new lyssavirus species has been continuously updated as new information becomes available (discussed in Section 2.2) and currently takes into consideration the genetic distance of the complete N gene with a threshold of between 80-82% nucleotide identity or 80% nucleotide identity for the concatenated coding regions of all five known lyssavirus genes. There is also a need for consistency in phylogenetic trees that have been obtained using various evolutionary models (Rupprecht et al., 2017, Dietzgen and Kuzmin, 2012b). The need for such genetic characterisation often requires extensive testing and thus ample genetic material. Thus to obtain enough genetic material for the future testing associated with virus characterisation, isolation of the virus is crucial. With the use of cell tissue culture not always possible, animal models offer the opportunity to successfully isolate novel virus for full genome characterisation as well as specific research disciplines that include the evaluation of biological products, pathogenicity studies and an alternative in absence of siotable cell tissue culture (Markotter et al., 2009; Faber et al., 2009; Bourhy et al., 1989). With improvements in sequencing technology, it is also becoming easier and more cost-effective to determine full genome sequences in a timely manner allowing for more extensive genome analysis (Barba and Hadidi, 2017; Marston et al., 2013). The use of next generation amplicon sequencing allows for the genome sequence to be obtained using fewer amplified products at a much cheaper rate. The lyssavirus genome is a negative, non-segmented single-stranded RNA genome and the virion is usually 130-250 nm in length and 60-100 nm in diameter (Rupprecht et al., 2017; Johnson et al., 2010b). The genome is roughly 12 kb long and encodes five proteins that are needed for virus survival and replication. The proteins are the nucleoprotein (N), phosphoprotein (P), matrix protein (M) and a RNA dependent polymerase (L) and are conserved across all Rhabdoviridae (Chaitanya et al., 2019; Dietzgen and Kuzmin, 2012b). Each gene contains a transcription initiation and termination signal as well as promotor sequences that initiates replication of the individual proteins, present in the 3’

leader and 5’ tailing regions. The proteins are separated by intergenic regions and include untranslated regions (UTRs) harbouring important signals that are used for the transcription and replication and also have an effect on gene transcription and vary between the different lyssavirus species (Chaitanya et al., 2019; Rupprecht and Nagarajan, 2015; Arguin et al., 2002; Tordo et al., 1988). These genomic characteristics are very important when comparing lyssavirus genomes and are used to understand virus phylogeny, the functionality of genes and what they are used for, and also to provide insight of the antigenic diversity of the virus proteins when assessing the amount of cross reactivity between virus species to establish if commercially available rabies biologicals will provide a degree of protection (Rupprecht et al., 2017; Hanlon et al., 2005; Badrane et al., 2001).

This chapter describes the isolation of the Matlo bat lyssavirus (MBLV) in the insectivorous bat species, Natal long-fingered bat (*Miniopterus natalensis*). This will include the isolation of MBLV, subsequent full genome analysis and characterisation through the use of next generation amplicon sequencing and the determination of the genomic termini by circularisation of the genome followed with PCR amplification, cloning and sequencing of the required genome termini.

4.1 Materials and Methods

4.1.1 Virus isolation

For the confirmed lyssavirus positive brain sample (UP 5619), virus isolation was conducted in collaboration with Dr. Claude Sabeta (Agricultural Research Council-Onderstepoort Veterinary Research Institute, Onderstepoort, South Africa (ARC-OVR)) as part of routine rabies diagnostic activities. This was done by preparing remaining brain material as a 20% tissue suspension in 1 ml Phosphate Buffered Saline (PBS) (Lonza, Switzerland) followed by centrifugation for 10 minutes at 500 g. The mice that were used for virus isolation were 3-day old suckling mice (Onderstepoort Biological Products, South Africa). For inoculation, 0.5 ml BD Micro-Fine™ Insulin Syringes (Becton Dickinson, United States of America) were used (Rupprecht et al., 2018). Mice were individually inoculated by placing the individual flat on a decontaminated work bench, grasping the mouse by the tail with the fourth and fifth finger and by the scruff using the thumb and second finger. This sufficiently immobilises the mouse and allows manipulation of the head. The syringe was then loaded with 0.20 uL brain/ PBS homogenate and pushed through the skull between the ear and the eye, just inside the central line as depicted in Figure 4.1.

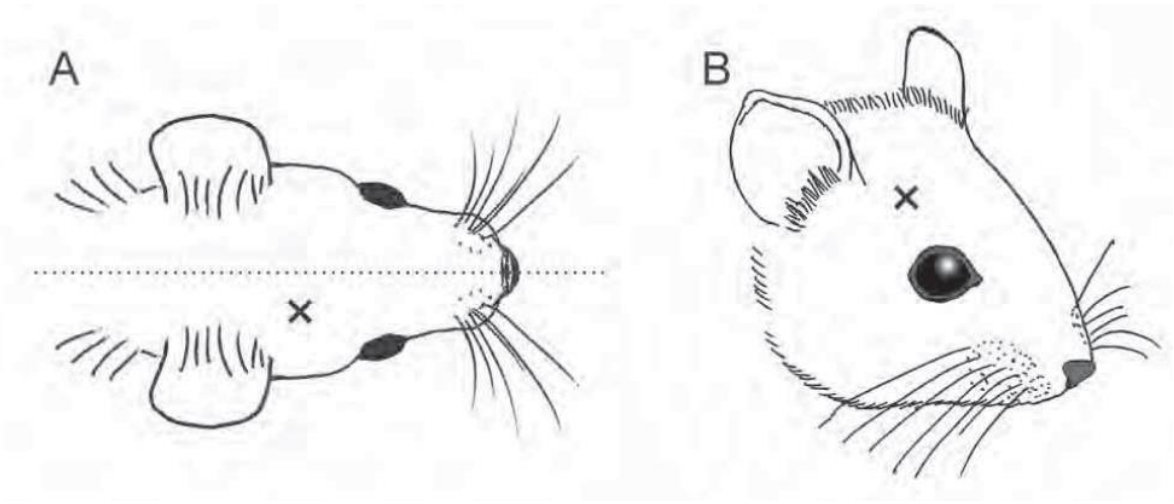


Figure 4.1. Illustration of placement of intracranial inoculation of suckling mice. A represents the dorsal view and B the facial view. This is an adaptation of an original work "Laboratory techniques in rabies. Geneva: World Health Organization (WHO); 1996; fifth edition (2018). Licence: ID: 352428. This adaptation was not created by WHO. WHO is not responsible for the content or accuracy of this adaptation. The original edition shall be the binding and authentic edition).

The needle was inserted roughly 3mm into the brain tissue. After the desired volume of inoculum was injected, the needle was slowly and gently removed to prevent any reflux of injected inoculum. The inoculated mice were then placed back with the mother and observed for two hours to ensure

no abnormal behaviour such as slow movements, ataxia, and continuous one-side rotation. The mice were observed for a total of 21 days with daily routine checks performed to ensure adequate water and food supply was available. The number of healthy, sick, and dead animals were also noted daily. Any animals that succumbed to disease were removed, placed in individual, sealable containers and frozen at -20 C. Any animals that did not succumb to disease by day 21, were euthanised by via intoxication with isoflurane as described by the WHO (Rupprecht et al., 2018). The removal of infected brain material was performed in a Biosafety level 3 facility by placing the carcass on clean absorbent material with the ventral surface facing downwards. The skin of the neck and head was pulled back and cut away using sterilised forceps and curved scissors. Thereafter the calvarium was cut away, the brain was removed from the skull using forceps and placed in a clean 1.5 ml microcentrifuge tube and frozen at -80 ºC. Confirmation of lyssavirus infection was performed using standardised rabies Fluorescent antibody test (FAT) protocols (OVR reference number- 509/18) (OIE, 2018). A polyclonal fluorescein isothiocyanate anti-lyssavirus conjugate (N4-18, Rabies unit, ARC-OVR, South Africa) was used, diluted to 1:100 in PBS ((Lonza, Switzerland), pH 7.4 (Lonza, Switzerland)) with Evans Blue (0.5% in PBS (Lonza, Switzerland)) as counterstain.

4.1.2 MiSeq amplicon sequencing primer design

For the full genome sequencing of MBLV, phylogroup specific primers were designed. The complete genomes of WCBV (Genbank accession: NC025377), LLEBV (Genbank accession: NC031955) and IKOV (Genbank accession: JX193798) were used to construct a multiple alignment. The primer sets were designed to allow amplicons to overlap by a minimum of 300 bp, covering the entire genome (Figure 4.2).

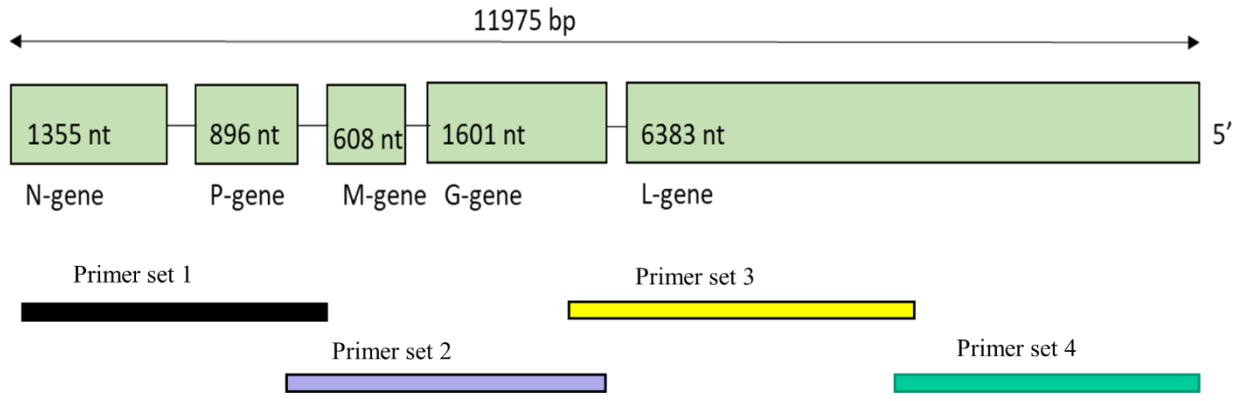


Figure 4.2. Schematic representation (not drawn to scale) for the design of amplicon sequencing primers, based on Phylogroup III reference sequences, covering the entire lyssavirus genome. Lengths depicted are that of WCBV (NC025377.1).

Alignments were performed using Bioedit's clustal W multiple alignment tool (Hall, 1999) and conserved regions covering the entire genome were identified. Differences observed in the alignment of the phylogroup III sequences were replaced with degenerate bases (Table 4.1).

Table 4.1. Summary of primers designed for miseq amplicon sequencing using phylogroup III lyssaviruses as references.

Primer name	Primer binding position	Melting temperature	Sequence 5'-3'
CG LYSSA S1 F	1-25	52.8 ºC	ACG CTT AAC ARC WAA AWM YYA KAA G
CG LYSSA S1 R	4031-4049	49.1 ºC	TRA ACA DBC CTC TYT CAT C
CG LYSSA S2 F	2771-2793	55.3 ºC	TCT GGB AAY MGA MGR ATG ATA GG
CG LYSSA S2 R	6941-6967	52.3 ºC	AYT TTT TCA TAT GGA CTT GAT CRT AMA
CG LYSSA S3 F	6347-6364	49.5 ºC	TRG AYT GGG ATG ARG ARA
CG LYSSA S3 R	9569-9587	46.9 ºC	CTV ACW GAG ATA TGA GACA
CG LYSSA S4 F	8671-8690	55.8 ºC	GAG GAY CCW ACC ACH CTS AA
CG LYSSA S4 R	11996-12016	54.3 ºC	ATC CAG TGA GCA GAC AAA CTT
001lys[a]	1-15	43.4 ºC	ACG CTT AAC GAM AAA
JW12[b]	55-74	48.6 ºC	ATG TAA CAC CYC TAC AAT G

Based on most closely related viruses'reference genomes
WCBV- NC025377, LLEBV-NC031955 and IKOV- JX193798
Primer binding position based on WCBV- NC025377
001lys and JW12 binding positions based on Pasteur virus sequence (M13215)
[a] (Markotter et al., 2006), [b] (Heaton et al., 1997)
Degenerate bases- **R**- A/G, **S**- G/C, **M**- A/C, **Y**- C/T, **H**- A/C/T, **V**- A/C/G, **M**- A/C**, W**- A/T, **B**- C/G/T, **D**- A/G/T, **K**- G/T

4.1.3 MiSeq amplicon amplification and sequencing

Full genome sequencing was performed using targeted amplicon sequencing on the MiSeq (Illumina, United States of America) platform using inoculated brain material from Section 4.1.1 (Marston et al., 2017). RNA was extracted as previously described in Section 3.2.1. Thereafter cDNA was made using random priming. For a 20 µl reaction volume, 1 µl of random hexamer

primers (100ng/µl) (Integrated DNA Technologies, South Africa) was combined with 5 µl total RNA, 1 µl of dNTP Mix (10mM) (Thermo Fisher Scientific, United States of America) and 6 µl nuclease-free water (Ambion, United States of America) to a total of 13 µl. This mixture was then heated to 65℃ for 5 minutes, incubated on ice for 1 minute and briefly centrifuged followed by the addition of 4 µl of first strand buffer (5X) (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$) (Thermo Fisher Scientific, United States of America), 1 µl of DTT (0.1M) (Thermo Fisher Scientific, United States of America), 1 µl RNase inhibitor (40 U/µl) (Thermo Fisher Scientific, United States of America) and 1 µl of SuperScript™ III RT enzyme (200U /µl) (Thermo Fisher Scientific, United States of America). This was mixed by pipetting followed by incubation at 25 ℃ for 5 minutes. The mixture was then incubated at 50℃ for 60 minutes followed by inactivation by heating at 70℃ for 15 minutes. The cDNA was stored at -20℃.

The PCR was set up as a 50 µl reaction for each primer set containing 10 µl Phusion HF buffer (5X) (New England Biolabs, United States of America), 1 µl dNTPs (10mM) (Thermo Fisher Scientific, United States of America), 2.5 µl of the forward primer, 2.5 µl of the reverse primer, 0.5 µl Phusion DNA Polymerase (New England Biolabs, United States of America) 1.5 µl dimethyl sulfoxide (DMSO) (New England Biolabs, United States of America) and 2 µl cDNA. The final volume was made up to 50 µl with nuclease-free water (Ambion, United States of America). The reaction was run at 98 °C for 30 seconds (initial denaturing) followed by 35 cycles at 98 °C for 5-10 seconds, 45-72 °C (dependant on primer set, Table 4.1) for 30 seconds and 72 °C for 2 minutes. Final Extension was performed at 72 °C for 10 minutes followed by a hold at 4 °C. Thereafter the products were separated on an agarose gel as described in Section 3.2.4. Bands of the correct length for each primer set were purified as described in Section 3.2.4 followed by determination of the concentration of the purified product using a Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, United States of America) in a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, United States of America) according to manufacturer's instructions. The purified product was then sent to The National Institute for Communical Diseases' (NICD) core sequencing facility where library preparation was performed for each amplicon using the Nextera XT library prep kit (Illumina, United States of America) according to manufacturur's instructions. The samples were then run on an Illumina MiSeq sequencing platform (Illumina, United States of America) with a 500X coverage.

Table 4.2 Annealing temperatures of primer sets used during miseq amplicon amplification PCRs.

Primer set	Amplicon size	Annealing temperature
CG LYSSA S1 F and CG LYSSA S1 R (Region 1)	4049 bp	45.9 °C
CG LYSSA S2 F and CG LYSSA S2 R (Region 2)	4167 bp	48.8 °C
CG LYSSA S3 F and CG LYSSA S3 R (Region 3)	3223 bp	43.2 °C
CG LYSSA S4 F and CG LYSSA S4 R (region 4)	3345bp	50 °C
JW12 and CG LYSSA S1 R (Region 1b)[a]	3994 bp	48 °C
CG LYSSA S4 F and 001lys (Region 4b)[b]	3626 bp	42 °C

Based on phylogroup III reference genomes
Genome size estimates based on WCBV- NC025377.1
[a, b] -Region 1b and region 4b were used as alternative amplification target regions after the initial amplification using regions 1 and 4 were not successful.

4.1.4 Lyssavirus genomic terminal sequence determination

4.1.4.1 Ligation of lyssavirus genome

To determine the sequences of the 3' and 5' genome ends, the viral genome was ligated and circularised using RNA ligase (Kuzmin et al., 2008). A mastermix of 2 µl T4 RNA Ligase (10 U/µl) (Thermo Fisher Scientific, United States of America), 1 µl RiboLock RNase inhibitor (40 U/µl) (Thermo Fisher Scientific, United States of America), 20 µl Polyethylene glycol-8000 (40% PEG8000) (Merck, United States of America), ligase reaction buffer (10X)(500 mM Tris-HCl (pH 7.5 at 25 °C), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP) (Thermo Fisher Scientific, United States of America), 4 µl bovine serum albumin (BSA) (1mg/µl) (Thermo Fisher Scientific, United States of America) and 4 µl template RNA was prepared in a 0.2 ml thin-walled PCR tube followed by overnight incubation at 16 ºC. Thereafter, ethanol precipitation was performed by transferring the reactions to a 1.5 ml microcentrifuge tube and adding 4 µl NaAOH (3 M) and 80 µl ice cold ethanol (100%, molecular grade) to each reaction followed by incubation at -80 ºC for 1 hour. The reactions were then incubated at room temperature for 30 minutes followed by centrifugation at 16 000 g for 30 minutes. Thereafter the supernatant was carefully removed, the pellet washed with 500 µl ice cold 70% ethanol (Merck, United States of America) (allow the pellet to soak for 2 minutes) and then centrifuged at 16 000 g for 10 minutes. The wash step was then repeated.

Thereafter the pellet was allowed to airdry and the RNA pellet was resuspended in 15 µl nuclease free water (Ambion, United States of America).

4.1.4.2 Amplification of lyssavirus genome ends

For the amplification of the genome ends, specific nested RT-PCR primer sets were designed, with the forward primer binding near the 5' end and the reverse primer binding near the 3' end of the lyssavirus genome as shown in Figure 4.3. The sequences obtained during Miseq amplicon sequencing were used as reference for primer design (Table 4.3).

Table 4.3 Summary of primers designed for lyssavirus genomic terminal sequence determination using MiSeq amplicon sequencing data as a reference.

Primer name	Primer binding position	Melting temperature	Sequence 5'-3'
CGL51F	11943-11964	49.1 ºC	CAA TCA GCC AGT TAC ATA TTA C
CGL52F	12206-12227	47.7 ºC	GAA CTT CTC TTC TAG AAT ACA G
CGL31R	250-270	49.7 ºC	GAT CCA ATT TAG CTG CAT TG
CG32R	105-125	50 ºC	GCT TGA GAG TCA CTA TTT CAT

Primer binding position aligned using obtained Miseq amplicon sequences aligned with WCBV-NC025377

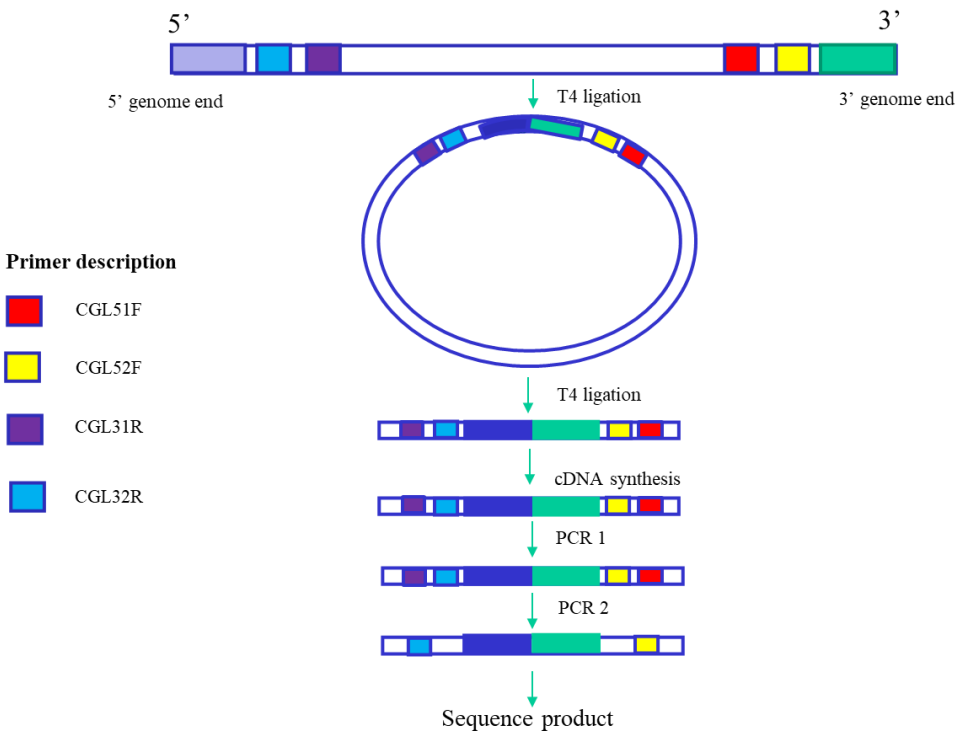


Figure 4.3 Schematic representation of the method used for sequence determination of lyssavirus 3' and 5' genomic ends using RNA ligase.

Thereafter reverse transcription was performed by adding 1 µl of CGL51F primer (10 pmol)(Table 4.3), 1 µl dNTP Mix (10 mM) (Thermo Fisher Scientific, United States of America), 5 µl of ligated RNA and 3 µl of nuclease-free water (Ambion, United States of America) to a 0.2 ml thin-walled PCR tube. The contents were briefly centrifuged and then incubated at 65 °C in for 5 minutes. The tube was placed on ice for 1 minute. A master mix containing 2 µl First-strand Buffer (5X)(250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$) (Thermo Fisher Scientific, United States of America), 2 µl of Dithiothreitol (DTT) (0.1 M) (Thermo Fisher Scientific, United States of America), 1 µl Ribolock RNase inhibitor (40 U/µl) (Thermo Fisher Scientific, United States of America), 4 $MgCl_2$ (25mM, Thermofischer, United States of America) and 1 µl SuperScript™ IV RT enzyme (200 U/µl) (Thermo Fisher Scientific, United States of America) was added to each tube and incubated at 50 °C for 50 minutes followed by inactivation at 85 °C for 5 minutes using a dry heating block (Labnet International, Inc, United States of America). The cDNA was then stored at -20 °C.

The first PCR was set up as a 50 µl reaction containing 0.25 µl Dream Taq Polymerase (5U/µl) (Thermo Fisher Scientific, United States of America), 5 µl Dream Taq PCR Buffer (10x) (containing KCl, $(NH4)_2SO_4$ and 20 mM of $MgCl_2$) (Thermo Fisher Scientific, United States of

America), 1 µl dNTPs (10mM) (Thermo Fisher Scientific, United States of America), 1.25 µl of the forward primer (CGL51F) (10 pmol), 1.25 µl of the reverse primer (CGL31R) (10pmol), and 5 µl template cDNA. The final volume was made up to 50 µl with nuclease-free water (Ambion, United States of America). The reaction was run at 95 °C for one minute (initial denaturing) followed by 40 Cycles at 95 °C for 30 seconds, 48 °C for 30 seconds and 72 °C for 1 minute. Final Extension was performed at 72 °C for 5 minutes followed by a hold at 4 °C. Thereafter the products were run on an agarose gel as described in Section 3.2.4. Bands of the correct length were purified as described in Section 3.2.4.

The amplified product obtained during the first PCR was used as template for the nested PCR reaction. The nested PCR was set up as a 50 µl reaction containing 0.25 µl Dream Taq Polymerase (5U/µl) (Thermo Fisher Scientific, United States of America), 5 µl Dream Taq PCR Buffer (10x) (containing KCl, (NH4)2SO4 and 20 mM of $MgCl_2$)(Thermo Fisher Scientific, United States of America), 1 µl dNTPs (10mM) (Thermo Fisher Scientific, United States of America), 1.25 µl of the forward primer (CGL52F) (10 pmol), 1.25 µl of the reverse primer (CGL32R) (10pmol), and 5 µl first round PCR product. The final volume was made up to 50 µl with nuclease-free water (Ambion, United States of America). The reaction was run at 95 °C for one minute (initial denaturing) followed by 40 cycles at 95 C for 30 seconds, 48 °C for 30 seconds and 72 °C for 1 minute. Final extension was performed at 72 °C for 5 minutes followed by a hold at 4 °C. Bands of the correct length were purified as described in Section 3.2.4.

4.1.4.3 Ligation and transformation

The pGEM-T Easy® Vector system (Promega, United States of America) was used for cloning of the purified nested PCR product (Section 4.1.4.2). The formula (ng insert to add = ((ng of vector x kb size of insert) / (kb size of vector)) x (insert molar ratio/vector molar ratio) was used to calculate the amount of insert needed and cloning attempts were performed at 1:3 vector to insert ratio (as recommended by the manufacturer). Thereafter, ligation and transformation were performed according to the manufacturers' instructions with minor modifications. The ligation reactions were set up in 0.2 ml thin-walled PCR tubes by combining the following: 1x rapid Ligation Buffer (10x; Promega, United States of America), 1 µl pGEM-T Easy® Vector (50 ng/µl; Promega, United States of America), 25 ng of PCR product, 1 µl of T4 DNA Ligase (3 Weiss units/µl; Promega, United States of America) and nuclease-free water (Ambion, United States of America) to a final volume of 10 µl. For the positive control reaction, 1 µl of control insert DNA

(4 ng/µl, Promega, United States of America) was used instead of the sample DNA. For the background control, no insert or vector was added. The reactions were mixed by gentle pipetting and were incubated overnight for 18 hours at 4 °C. Luria Bertani (LB) agar plates supplemented with ampicillin (100 ng/ml; Zymo Research, United States of America) were prepared by mixing 35g of Vegitone LB Agar (Sigma- Aldrich, United States of America) with 1L of distilled water. The mixture was then autoclaved at 121 °C for 20 minutes and allowed to cool to below 50 °C. Thereafter, 1 ml ampicillin (100 ng/ml; Zymo Research, United States of America) was added, and plates were subsequently cast. Plates were left to solidify and then stored at 4 °C until use. Plates were allowed to reach room temperature before a 100 µl of Isopropyl-D-1-thiogalactopyranoside (IPTG, 0.1M; Thermo Fisher, United States of America) and 50 µl of X-Gal Solution (20 mg/ml; Thermo Scientific, United States of America) were spread on each plate and incubated at 37 °C for 30 minutes. The tubes containing the ligation reaction were then briefly centrifuged for 5 seconds at 500 x g. For the transformation reaction, 2µl of each ligation reaction was added to a 1.5 ml microcentrifuge tube on ice. High-efficiency competent JM109 cells (>10^8 cfu/µg; Promega, United States of America) were placed on ice to thaw. To each ligation aliquot, 50 µl of competent cells were added and placed on ice for 20 minutes. The cells were then placed in a dry heating block (Labnet International, Inc, United States of America) for 45 seconds at 42 °C and then returned to ice for 2 minutes. Thereafter, 950 µl of super optimal broth with catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; Invitrogen, United States of America) was added to each reaction tube and were placed in an Ectron shaking incubator (Infors HT, United States of America) at 37 °C and 150 rpm for 90 minutes. An aliquot of 100µl of each of the sample reactions was plated in duplicate onto the LB-Ampicillin/IPTG/X-Gal agar plates. A single plate was also included for the positive and background control respectively. Plates were incubated overnight at 37 °C in a Series 2000 incubator (Scientific, United States of America) and thereafter stored at 4 °C until colonies were selected.

4.1.4.4 Colony PCR

Blue-white selection was performed on each plate to select colonies that appeared to contain the expected insert (white colonies). For each sample, seven colonies were selected with a sterile pipette tip and transferred into individual 0.2 ml thin-walled PCR tubes to which 50µl of nuclease-free water (Ambion, United States of America) was added. The tubes were then incubated at 95 °C for 10 minutes and subsequently centrifuged at 12 000 x g for 5 minutes. After centrifugation,

tubes were handled with care not to disturb the pellet. Reagents for the colony PCR master mix were thawed on ice and briefly centrifuged for 5 seconds at 500 x g. A master mix containing 1 µl M13 forward primer (10pmol, Integrated DNA Technologies; 5'-GTA AAA CGA CGG CCA G-3'), 1.25 µl M13 reverse primer (10pmol, Integrated DNA Technologies; 5'-CAG GAA ACA GCT ATG AC-3'), 2.2 µl dNTP mix (10mM, Thermo Scientific, United States of America), 1X DreamTaq™ buffer (10X, Thermo Scientific, United States of America), 1.25 U DreamTaq™ polymerase (5U/µl, Thermo Scientific, United States of America) and nuclease-free water (Ambion, United States of America) to a final volume of 45 µl per sample was prepared. In 0.2 ml thin-walled PCR tubes, 5 µl of each colony supernatant was added to 45µl of master mix followed by incubation in an Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, United States of America) under the following conditions: 94 °C for 5 minutes; 40 cycles of 94 °C for 30 seconds, 48 °C for 30 seconds and 72°C for 90 seconds; final extension at 72 °C for 7 minutes and 4 °C hold. Amplified products were then analysed with agarose gel analysis as described in Section 3.2.4. Amplicons were inspected to ensure the presence of the correct insert size, purified and sequenced as described in Sections 3.2.4 and 3.2.5, with the M13 forward primer to confirm the presence of the genome ends sequence. The raw sequences generated were analyzed with BioEdit Alignment Editor Version 7 (Hall, 1999). Manual base-calling was done where needed and nucleotide sequences trimmed. The obtained sequences were then compared to all other known lyssavirus sequences (Appendix C1) using the Clustal W multiple alignment function in the BioEdit software (Hall, 1999).

4.1.5 Full genome assembling and phylogenetic analyses

Sequencing results were imported into the CLC Main Workbench 6 (CLCBio), where sequences were assembled using de novo assembly. The assemblage of the reads performed on the CLC genomics workbench included the quality control of obtained reads, the removal of library preparation adapters and the removal of host sequences by mapping the acquired reads to an available full genome of the closest related lyssavirus, WCBV (Genbank accession: NC025377). Thereafter the assembled sequences were imported into the NCBI open reading frame finder where genes were assigned. The sequences were then exported into the BioEdit software (Hall, 1999). The obtained sequences were then compared to all other known lyssavirus sequences using the Clustal W multiple alignment function in the BioEdit software (Hall, 1999). Phylogenetic relationship comparison of the sequences was performed using Bayesian inference (BEAST package) to determine the relatedness of the viral gene sequences to those of other known

lyssaviruses (Drummond et al., 2012). Reference sequences used during phylogenetic comparison were specifically chosen to include all known lyssavirus diversity allowing for a representative analysis. Reference sequences were also chosen to include the specific diversity that has been detected in (Appendix C1). The aligned sequences were analysed using the JModel test software v.2.1.7 to find the best-fit DNA substitution model using the Bayesian Information Criterion (BIC) (Darriba et al., 2012). Bayesian Evolutionary Analysis Sampling Trees (BEAST) software v1.8.4 using the general time-reversible substitution model plus invariant sites and gamma distribution, an underlying coalescent process with constant population size along with the Bayesian MCMC chains set at 10 000 000 repeats sampling every 1 000 trees was used (Drummond et al., 2012). Trees were generated using Tree annotator (v10.1.4) with a burn in value of 10%. Thereafter viewing and editing of trees was performed using Figtree v1.4.0. For further phylogenetic analyses, MEGA X was also used employing the maximum likelihood method with a 1000 bootstrap replicates using the general time-reversible substitution model plus invariant sites and gamma distribution (Kumar et al., 2018).

4.1.6 Full genome sequence comparison

Full genome comparison was performed using nucleotide as well as amino acid similarity analyses between lyssaviruses. Gene sequences obtained from the sample were translated into amino acid sequences (in the correct reading frame) and aligned using the the Clustal W multiple alignment function in the BioEdit software (Hall, 1999). The nucleotide and amino acid similarity plots were generated for the concatenated coding region of the full genome of MBLV and the selected reference lyssavirus genomes (Appendix C1). For the analyses of the five specific genes, alignments for each gene as well as for the UTRs and genomic terminal sequences were performed using BioEdit software (Hall, 1999). The nucleotide similarity matrixes for the full genome as well as individual genes and UTRs were generated from the alignments in the BioEdit software. Pairwise similarities between viral sequences were determined and analysed in MEGA X with pairwise deletions (Kumar et al., 2018).

4.2 Results

4.2.1 Virus isolation

The two insectivorous bat samples that tested positive for lyssavirus nucleic acids in the previous section contained very little brain material for further testing. Sample UP 6246 was already depleted during the initial screening. We performed a mouse inoculation test with the remaining brain material of UP 5619 using 3-day old suckling mice to ensure sufficient material was available for further molecular characterization and to obtain a viable isolate of the virus. There were no deaths noted during the first five days after inoculation. The first death was observed on day six and by the tenth day, eight mice had succumbed to disease. On day 13, the last mouse deaths were noted. The remaining three mice were euthanised on day 21. Thereafter, all mice were necropsied, and the recovered brain material was subjected to a FAT. The FAT was performed on brain material of mice infected with UP 5619 along with a positive control (CVS-11, Genbank accession number M13215) and a negative control, using the polyclonal fluorescein isothiocyanate anti-lyssavirus conjugate (N4-18, Rabies unit, ARC-OVR, South Africa) at a 1:100 dilution. Positive green fluorescing antigen was observed in 100% of the microscopic fields of the positive control and UP 5619 and none in the negative control.

4.2.2 Miseq amplicon amplification and sequencing

Amplification of amplicon regions 1-4 was initially conducted using the annealing temperatures described in Table 4.2. Regions two and three were successfully amplified without the need to troubleshoot or adjust PCR conditions and produced the expected band sizes of 4167 bp and 3225 bp respectively. Regions 1 and 4 were not successfully amplified when the selected primer sets were used. Additionally, primer sets for region 1b and region 4b were used and allowed for successful amplification of the desired regions and produced the expected band sizes. All four regions were successfully purified followed by determination of the concentration of the purified product to ensure sufficient DNA material for Illumina MiSeq next generation sequencing (Table 4.4).

Table 4.4 Summary of the DNA concentration of each genome region amplified during full genome amplification as well as the amounts of reads received after Illumina MiSeq next generation sequencing at the The National Institute for Communical Diseases' (NICD) core sequencing facility.

Genome region	DNA product concentration	Reads received after amplicon sequencing
Region 1b	12.90 ng/µl	312823 reads
Region 2	34.00 ng/µl	512067 reads
Region 3	21.00 ng/µl	429706 reads
Region 4b	15.20 ng/µl	665217 reads

4.2.3 Lyssavirus genomic terminal sequence determination

After miseq amplicon sequencing failed to amplify the genomic terminal sequences of the genome of MBLV, a method involving genome circularisation via ligation and cloning were attempted (Kuzmin et al., 2008). The genome ligation, cDNA synthesis and first round PCR procedures were performed as described in Section 4.1.4. First round PCR product was visualised by agarose gel electrophoresis but did not produce bands of the expected size. Second round PCR was performed using the first round product and produced the expected size product. Seven white colonies were chosen during blue/white selection and subjected to colony PCR reactions including a positive control and background negative control. The expected size band (850 bp) was observed for all selected colonies and was successfully sequenced producing the genomic terminal sequence for the 5' and 3' ends of the MBLV genome.

4.2.4 Phylogenetic analysis

The phylogeny of the gene specific and full genome lyssavirus sequences obtained in this study was compared and analysed with publicly available sequences representing lyssaviruses from all species within the *Lyssavirus* genus. The best-fit DNA substitution model that was used in the analyses of individual genes, as well as the full genome, was determined to be the generalised time reversible model with a gamma distribution and invariant sites (GTR+I+G). The phylogenetic analyses of MBLV P-, M-, G-, and L-genes as well as the concatenated coding regions of all genes provided similar tree topology, with a posterior probability of 1 and bootstrap value of 100, when using Bayesian phylogeny and maximum likelihood analyses. As similar tree topology was seen when comparing all the different genes analysed, Bayesian phylogeny and maximum likelihood analyses trees for the P-, M-, G- and L-gene can be found in Appendix D1-8. As was expected following full N gene analysis (chapter 3), phylogenetic analyses of lyssavirus nucleotide sequences revealed the formation of two distinct phylogroups. The full genome (concatenated

coding regions) sequence grouped outside of the two phylogroups and formed a clade with the unclassified lyssaviruses (Figure 4.4 and Figure 4.5).

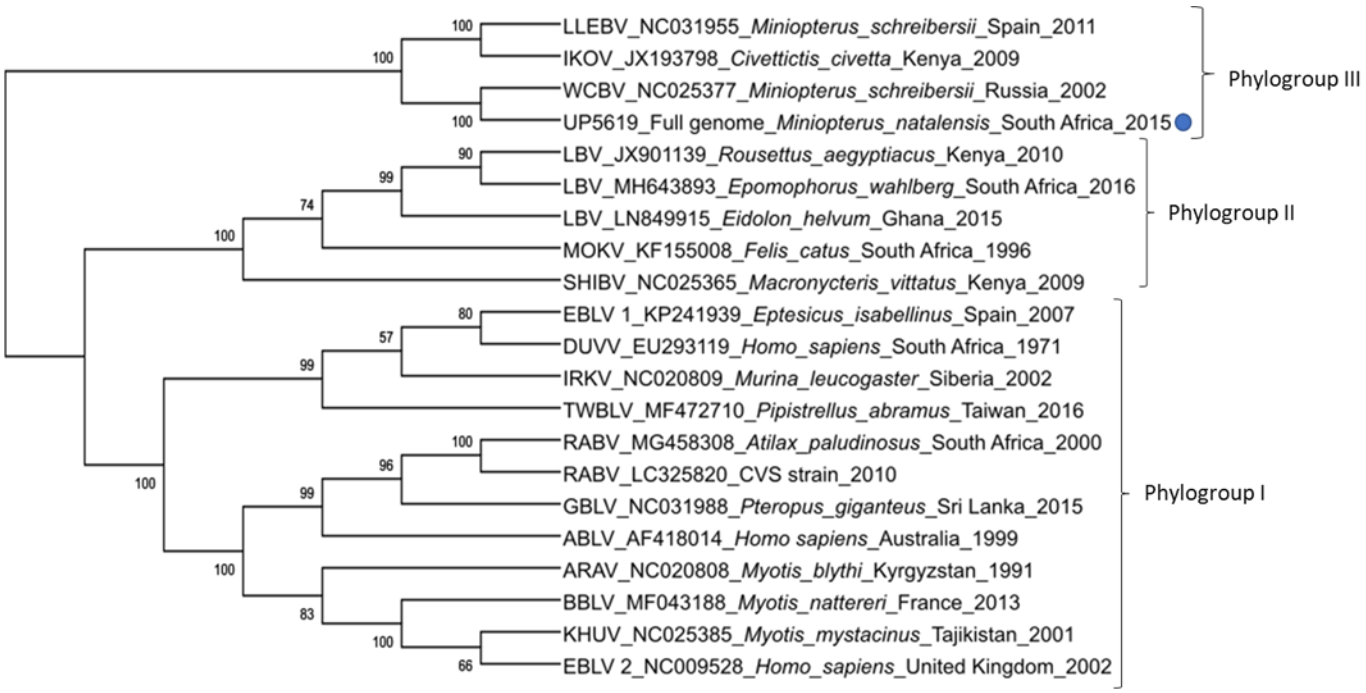


Figure 4.4 Maximum likelihood analysis of the relatedness of representative lyssaviruses and MBLV (blue circle), Phylogeny was constructed using MEGA X using complete concatenated genomes, GTR+G+I model with a bootstrap of 1000 replicates. Only bootstrap values above 50 are shown.

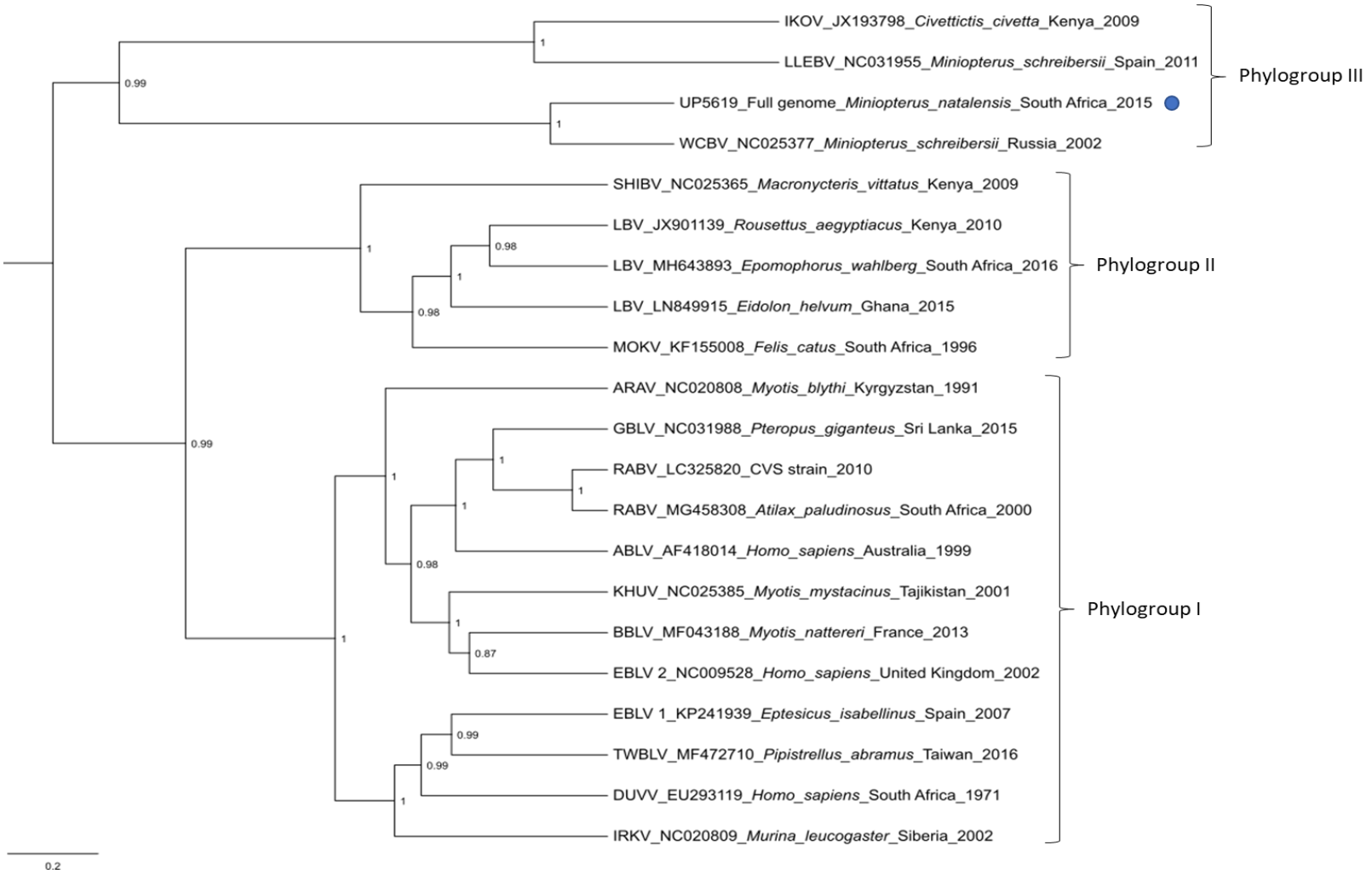


Figure 4.5 Phylogenetic analyses of MBLV (blue circle) full concatenated genome sequence and representative lyssaviruses. Bayesian phylogeny of all concatenated coding regions of the lyssavirus gene sequences was achieved using BEAST employing the GTR+I+G substitution model for posterior probabilities at MCMC chain setting of 10M samples every 1000 states.

The tree topology obtained when analysing the phylogenetic relatedness of MBLV using full concatenated genome sequence (as well as individual genes (Appendix D1-8)), revealed similar results as with the conserved full N gene when using maximum likelihood analyses (Figure 4.4) as well as Bayesian phylogeny (Figure 4.5). Both the Bayesian phylogeny as well as maximum likelihood analyses showed MBLV to group within the phylogroup III clade, most closely related to WCBV, with nodal support of 100 (Figure 4.4) and posterior probability of 0.99 (Figure 4.5). Full concatenated genome (and individual genes (Appendix D1-8)) tree topology clearly indicates the formation of a sister clade between MBLV and WCBV with a bootstrap value of 100 and a posterior probability of 1. This formed sister clade groups with the unclassified lyssaviruses along with IKOV and LLEBV but clearly splits into two separate sister clades (Figure 4.4 and Figure 4.5).

4.2.5 Full genome characterisation

The genomic characteristics of MBLV were established by determining the lengths of the specific gene sections, including the gene coding regions, intergenic region and genomic terminal ends (Table 4.5), amino acid composition (Table 4.5), nucleotide and deduced amino acid sequence identities (Table 4.7), intra- and interspecies sequence similarities (Figure 4.6; Appendix E1-2) as well as gene transcription initiation and termination sequences (Table 4.6).

Table 4.5 Summary of the genome characteristics of MBLV, obtained after full genome sequencing and assembly, comparing gene lengths in nucleotide bases to three other phylogroup III lyssaviruses.

	3' UTR	N GENE	N CDS	N-P IGS	P GENE	P CDS	P-M IGS	M GENE	M CDS	M-G IGS	G GENE	G CDS	G-L IGS	L GENE	L CDS	5' UTR
UP 5619 MBLV	58	1395	1353 (451)	4	1041	894 (298)	2	768	609 (203)	39	2304	1578 (526)	101	6509	6384 (2128)	57
WCBV NC 025377	58	1395	1353 (451)	4	1042	894 (298)	2	768	609 (203)	39	2303	1578 (526)	100	6510	6384 (2128)	57
LLEBV KY 006983	58	1398	1353 (451)	4	957	870 (290)	5	772	609 (203)	21	2146	1578 (526)	38	6462	6381 (2127)	70
IKOV JX 193798	58	1397	1353 (451)	4	957	870 (290)	4	781	609 (203)	23	2104	1575 (525)	39	6465	6381 (2127)	70

Number of amino acids indicated in brackets
UTR- Untranslated region, CDS- Coding region, IGS- Intergenic sequences

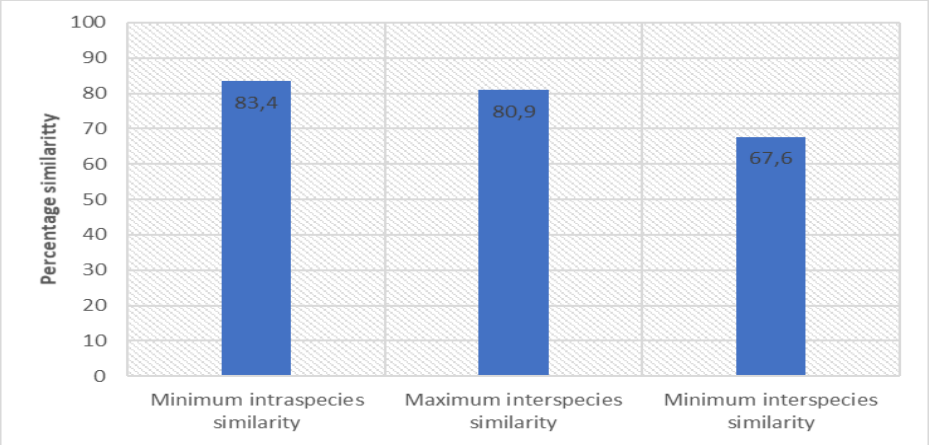
Table 4.6 Summary of the transcription initiation, termination and intergenic sequences of MBLV compared to that of WCBV.

Region	Transcription termination signal (TTS) MBLV/WCBV	IGS MBLV/WCBV	Transcription initiation signal (TIS) MBLV/WCBV
3' Leader-N	-	-	AACACCCCT/ AACACCCCT
N-P	TGAAAAAAA/ TGAAAAAAA	CATC/ CATC	AACACCCCT/ AACACCCCT
P-M	TGAAAAAAA/ TGAAAAAAA	CT/CT	AACACCCCT/ AACACCCCT
M-G	TGAAAAAAA/ TGAAAAAAA	39 nt / 39 nt	AACATCCCT/AA CATCCCT
G-L	TGAAAAAAA/ TGAAAAAAA	101 nt/ 100 nt	AACACCTCT/AACACCCCT
L- 5' Trailer	TGAAAAAAA/ TGAAAAAAA	-	-

Table 4.7 Nucleotide and deduced amino acid sequence identities of the coding regions all five lyssavirus genes of MBLV genome compared to other known lyssavirus species.

	Full Genome CDS nt	N nt	N aa	P nt	P aa	M nt	M aa	G nt	G aa	L nt	L aa
WCBV (NC02537)	78.9 %	80.9 %	95.5 %	75.2 %	83.2 %	84 %	96.5 %	76.4 %	86.6 %	79.1 %	92.7 %
IKOV (JX193798)	64.1 %	71.6 %	80.2 %	53.6 %	45.3 %	67.9 %	73.3 %	55.2 %	48.2 %	66 %	71.9 %
LLEBV (NC031955)	64.2 %	71.4 %	81.5 %	52.1 %	45.9 %	70.1 %	75.3 %	54.2 %	47.5 %	66.3 %	72.7 %
SHIBV (NC025365)	66.3 %	73 %	83.5 %	50.9 %	43.9 %	71.1 %	78.8%	56 %	53.2 %	69.2 %	76.5 %
MOKV (KF155008)	65.7 %	71.5 %	82 %	51.9 %	43.9 %	69.4 %	74.8 %	57.1 %	52.6 %	68.1 %	75.3 %
LBV (LN849915)	65.7 %	73.9 %	83.7 %	50.3 %	42.6 %	71.7 %	76.3 %	55.9 %	54.5 %	68.1 %	75.7 %
ARAV (NC020808)	65.4 %	72.6 %	83.3 %	50.6 %	45.8 %	68.8 %	77.3 %	53 %	50.1 %	68.8 %	76.1 %
BBLV (MF043188)	65.6 %	72.4 %	81.5 %	51.5 %	43.8 %	68.8 %	77.8 %	54.5 %	50 %	68.3 %	76 %
ABLV (AF418014)	65.2 %	72.9 %	82.6 %	49.4 %	43.5 %	69.7 %	76.3 %	54.1 %	51.6 %	67.7 %	74.6 %
DUVV (EU293119)	65.7 %	73.8 %	84.2 %	52 %	42.9 %	68.3 %	76.3 %	53 %	48 %	68.8 %	75.4 %
EBLV 1 (KP241939)	65.5 %	71.2 %	82.4 %	50.4 %	44.5 %	70.7 %	77.8 %	52.8 %	49.2 %	68.9 %	75.7 %
EBLV 2 (NC009528)	65.7 %	70.9 %	80.7 %	53 %	44.5 %	68.9 %	77.3 %	54.7 %	49.8 %	68.6 %	76 %
GBLV (NC031988)	65.1 %	70.5 %	83.1 %	49.7 %	45.5 %	70.2 %	78.3 %	54.5 %	51.3 %	68.4 %	75.3 %
IRKV (NC020809)	65.7 %	71.9 %	83.3 %	52.9 %	42.9 %	71.2 %	77.8 %	54.4 %	51.3 %	68.2 %	75.7 %
KHUV (NC025385)	65.4 %	72.3 %	81.3 %	51 %	44.5 %	67.8 %	78.8 %	54.4 %	50.3 %	68.3 %	76.2 %
RABV (LC325820)	64.6 %	71.8 %	81.7 %	52 %	42.5 %	68.1 %	75.3 %	55.1 %	51.1 %	67.4 %	75.1 %

Figure 4.6 Minimum intra-species and maximum inter-species sequence identities among 28 lyssaviruses for the conserved Nucleoprotein gene



Nucleotide and amino acid identities are shown in Table 4.7. The genome of MBLV were determined to be 12 278 nucleotides in length. The maximum nucleotide and amino acid identities for all five genes was with WCBV with a complete coding genome similarity of 78.9 %. The transcription termination signal (TTS) of all the genes was determined to be 5'-TGAAAAAAA-3' which is not surprising as the TTS is well conserved across all lyssaviruses (Delmas et al., 2008; Marston et al., 2007). The highest N gene coding region identities was determined to be with WCBV with 80.9 % for nucleotide sequence and 95.5% for amino acid sequence identity. When comparing the similarities between inter- and intra-species nucleotide identities of the conserved N gene of a set of known lyssavirus species (Appendix E1-2), MBLV equals the maximum interspecies similarity shown for the N gene with WCBV (80.9 %). When comparing the minimum intraspecies similarity of the N gene, MBLV shows 2.5% more nucleotide difference when excluding LBV lineages that have shown overlaps when comparing different lineages of the species and complicating species demarcation for very closely related virus species. The IGS between the N and P gene was established to be four nucleotide bases long (5'-CATC-3') al shown to be identical to that of WCBV. The TIS for the P gene was 5'-AACACCCCT- 3' and the highest P gene nucleotide and amino acid identities were established to be 75.2 % and 83.2 % with WCBV. The IGS between the P and M gene was determined to be only two nucleotide bases long (5'-CT-3') which was identical to that all lyssavirus species. The M gene coding region was found to be 609 bp (203 amino acids) long with the highest coding region identities being 84 % for nucleotide identify and 96.5 % for amino acids with WCBV. The M gene TIS was also shown to be identical to the P gene initiation signal with the M-G IGS being 39 bp long. The highest G gene coding region identities were also determined to be with WCBV with 76.4 % for nucleotide sequence and 86.6 % for amino acid sequence. The G gene TIS was identified as 5'-AACATCCCT-3' which

was identical to that of WCBV and the G-L IGS of 101 bp which showed a difference of one nucleotide with WCBV. The MBLV G-L IGS, together with WCBV, is the longest of all IGS of lyssavirus sequenced to date. It has been shown that the shorter the IGS, the more efficient the gene transcription. The long IGS present in MBLV and WCBV will contribute to lower expression of the L gene by attenuation (Kuzmin et al., 2008; Badrane and Tordo, 2001; Whelan et al., 2000). The L gene coding region was determined to be 6384 bp (2128 amino acids) long with maximum nucleotide and amino acid identities being 79.1 % and 92.7 % respectively. The TIS for the L gene was established as 5'-AACACCTCT-3'.

4.2.6 Pathogenic domain analyses

The glycoprotein (G), matrix protein (M) and phosphoprotein (P) was previously shown (discussed in Section 2.1) to be important genes involved in the pathogenicity of lyssavirus species. Multiple alignments of the G protein, M protein and P protein amino acid sequences obtained from MBLV were compared to other known lyssavirus species. Analyses were focused on specific amino acid regions that have previously been shown to be important for virus pathogenicity as well as known antigenicity sites (Table 4.8).

The alignment of the G protein amino acid sequences showed a significant number of residue similarities. The majority of the amino acid residue differences between WCBV and MBLV was determined to be present in regions not specifically associated with lyssavirus pathogenicity. Of the important amino acid residues highlighted in Table 4.8, all were identical to that of WCBV at the same amino acid positions. The important antigenicity sites (site II and III) were also found to be identical to that WCBV. Amino acid residues 133 (Trp), 330 (Ile) and 333 (Glu) substitutions were only present in the three unclassified lyssavirus species sequences.

When comparing amino acid residues implicated to be important for lyssavirus pathogenicity on the M gene, all but one residue between WCBV and MBLV were identical. Amino acid residue 81 was substituted from a Ser present in WCBV to an Asn in MBLV. Amino acid residues 35-38 forming the PPEY motif and residues 39-41 forming the VPL motif were both conserved across all lyssavirus sequences with the exception of SHIBV containing a substitution in the VPL motif.

The P protein alignment showed an amino acid substitution at position 147 when comparing MBLV and WCBV. The amino acid residues located at position 144-148 are relatively well conserved across phylogroup I and phylogroup II lyssavirus species, with the unclassified lyssaviruses showing multiple substitutions within this region.

Table 4.8 Comparison of important amino acid sites of the P-, M-, and G-gene between the newly identified lyssavirus (MBLV) and the other phylogroup III viruses

Gene	Amino acid Positions	Description of amino acid function	MBLV (UP5619)	WCBV (NC02537)	IKOV (JX193798)	LLEBV (NC031955)
Phosphoprotein Gene	144-148	aa involved in the phosphoprotein binding to the LC8 dynein light chain	DIAIQ	DIAVQ	QTDPI	QTEMH
Matrix Protein Gene	22-25	Important aa for vesicular stomatitis (related rhabdovirus) pathogenicity	PSAP	PSAP	APVL	ASAP
	35-38	Important aa for effective virion release and pathogenicity	PPEY	PPEY	PPEY	PPEY
	77	Involved in the disruption of the mitochondrion and induction of apoptosis.	R	R	R	R
	81		N	S	G	G
	95	Causes increased apoptosis if valine is replaced by an arginine.	V	V	I	V
Glycoprotein Gene	194	Causes increased viral spread, internalization and pathogenicity if asparagine is replaced by a lysine at aa position 194.	T	T	A	S
	198	Reduces pathogenicity if arginine is replaced by lysine at aa position 198.	K	K	I	R
	242	Important for pathogenicity of the Nishigahara strain.	S	S	S	S
	255		S	S	S	S
	268		L	L	I	L
	318	Important for neutrotrophin receptor (p75NTR) binding.	I	I	I	I
	330-333	Arginine or lysine at aa positon 330 is responsible for virulence in mice. A double mutation of arginine to lysine at amino acid positions 330 and 333 reduces virulence.	IKVE	IKVE	KSVD	KSVS
	352	Important aa for neutrotrophin receptor (p75NTR) binding.	Y	Y	H	Y

4.3 Discussion

In this chapter, we set out to isolate and characterise the full genome of MBLV identified in the previous chapter. With UP 6246 brain material already depleted after initial lyssavirus screening, it was essential to isolate the detected virus (MBLV) from the small amount of bat brain material left from UP 5619. As initial results implicated the possible detection of a novel lyssavirus, further analyses and characterisation were required as well as to have an available isolate for future studies thus the availability of sufficient testing material was required. It was decided to use suckling mice as opposed to cell tissue culture for viral isolation. This was done to allow the accumulation of high titre virus at the first inoculation as there was only a small amount of infected brain material available (Bourhy et al., 1989). The isolation of lyssaviruses using cell tissue culture also often takes multiple passages that could allow for the accumulation of genomic mutations (Bourhy et al., 1989). The ability of the virus to cause disease, although being through an unnatural route of infection, does highlight the importance of safety measures when working with possible infectious material as well as the need for virus pathogenicity studies.

MiSeq amplicon next generation sequencing was selected for full genome amplification as this method provided the ability to amplify large regions of the genome and also increase the chances of successful amplification (Marston et al., 2013). As the initial phylogenetic analyses grouped MBLV closest to WCBV, the primer sets were specifically designed using WCBV and the other two unclassified reference viruses to ensure the highest probability of successful annealing during PCR amplification. The inability of two of the initially designed primer sets to amplify their targeted regions of the genome supported the initial finding that this was a more divergent lyssavirus species. Two additional primer sets, targeting two different conserved regions, were used and successfully amplified the target regions.

The genomic characteristics of MBLV were determined to be most similar to that of WCBV, which included whole genome length, gene coding regions as well as intergenic sequence lengths. Specifically, the IGS region between the G and L gene for WCBV has been shown to differ significantly from all other known lyssavirus species. The typical length of this region varies between 19 and 29 nucleotide bases in other lyssavirus species with WCBV containing 100 nucleotide bases (Kuzmin et al., 2008; Marston et al., 2007). The IGS sequence length identified between the G-L genes for MBLV further supported the grouping of the virus closest to WCBV. As previously mentioned, the MBLV and WCBV G-L IGS is the longest of all IGS of lyssavirus.

The long IGS present in MBLV and WCBV will contribute to lower expression of the L gene by attenuation. The presence of more attenuated virus will allow for more time for virus transmission before the death of the infected host (Kuzmin et al., 2008; Badrane and Tordo, 2001; Whelan et al., 2000). When comparing the nucleotide and amino acid sequence identities along with the phylogenetic groupings' concatenated coding regions of the five lyssavirus genes', there is support that MBLV is a novel species. As described in Section 2.1, the current criteria for species demarcation take into consideration the genetic distance of the complete N gene with a threshold of between 80-82% nucleotide identity or 80% nucleotide identity for the concatenated coding regions of all five lyssavirus genes. Both the above-mentioned criteria are thus met with the highest N gene similarity being 80.9 % and the highest concatenated genome similarity being 78.9 %. This demonstrates adequate genetic variation present and the minimum genetic distance criteria required is met. When the intra- and interspecies minimum and maximum similarities were compared between described lyssaviruses, MBLV proved to have the same maximum interspecies similarity of 80.9 % when comparing the N gene coding region. This is similar to the same maximum interspecies similarity seen when comparing KHUV and BBLV. Intra- and interspecies similarities support the classification of MBLV as a new lyssavirus species.

The phylogenetic groupings of all five concatenated lyssavirus genes as well as whole genome sequences are supported by the nucleotide and amino acid sequence similarities. Phylogenetically, MBLV is grouped in the cluster of lyssaviruses forming phylogroup III, most closely associated with WCBV when using two phylogenetic evolutionary models applied on different genome regions. Strong bootstrap values of 90 and above were noted when comparing all genes, as well as the full concatenated genome of MBLV. This is also true when comparing the posterior probabilities obtained with strong support of above 0.9 noted. These nodal values further support the grouping of this virus closest to but separate from WCBV. The topology and nodal support obtained when comparing MBLV to other lyssavirus species groupings do provide support for MBLV to be classified as a novel lyssavirus species or a virus close related to WCBV. The phylogenetic groupings and nodal support obtained also demonstrates consistent phylogenetic topology using various evolutionary models which is the second criteria used by the ICTV. Comparing specific known amino acid regions that are known to influence the pathogenicity of lyssaviruses provides further support of the grouping of the newly identified virus (Table 4.8). The level of similarity between MBLV and the other unclassified lyssaviruses when comparing important antigenic regions provides initial evidence that cross neutralization will most probably not occur when immunised host are exposed to this virus (Horton et al., 2014; Weyer et al., 2008;

Hanlon et al., 2005; Nel et al., 2003). However, without specific serological cross-reactivity and animal challenge studies being performed, a definitive conclusion on the antigenic patterns, serological cross-reaction and vaccine protection cannot be made. This research will be conducted in the near future.

Chapter 5 – Final conclusion

This study reported on the detection of three lyssavirus positive samples that were obtained from insectivorous bats over the course of 16 years of surveillance across South Africa. This does indicate the presence and active circulation, albeit low, of lyssaviruses in insectivorous bat populations. The detection of a DUVV infection from a *N. thebaica* brings the total DUVV cases to six with three human fatalities. This is the second isolation of DUVV made from a *N. thebaica* bat, thus far the only bat species conclusively linked with this lyssavirus species (Markotter and Coertse, 2018). The *N. thebaica* species is known to be widely distributed throughout Africa and is also known to co-roost with various other bat species (ACR, 2019). This result together with the previous DUVV isolation made from *N. thebaica* further suggests that this bat species is a potential host for DUVV.

This study also reports the detection of a possible new lyssavirus, provisionally named Matlo bat lyssavirus (MBLV). Two isolations of MBLV were made from *M. natalensis* bats, sampled at two different cave systems in the northern regions of South Africa. These bats are known to be distributed throughout South Africa and they are primarily found in cave systems. *M. natalensis* is also a known migratory insectivorous bat, that has been known to often travel great distances and thus creating the opportunity for potential spillover events at multiple locations (ACR, 2019). However, to accurately determine if *M. natalenses* is the natural host of MBLV or if these infections are attributed to a spill-over infection from another host, the implementation of additional longitudinal surveillance, serological surveillance in this bat species as well as in other hosts sharing the same ecological niches must and will be implemented.

Based on phylogenetic analysis of all five lyssavirus genes and also the complete genome, MBLV belongs to the unclassified group of lyssaviruses and is most closely related to WCBV, with nucleotide identity ranging between 75.2-84% for the individual genes and 78.9 % for the whole genome. The obtained nucleotide identities fall within the species demarcation criteria as determined by the International Committee for the Taxonomy of Viruses. Should MBLV not be classified as a new lyssavirus species, it would represent a diverse lineage of WCBV, as there have been reports of WCBV seropositivity, or a virus closely related to WCBV, circulating based on the previous detection of VNA in *Miniopterus* bats sampled in Kenya (Kuzmin et al., 2008). This

could indicate that MBLV may potentially be found throughout the African continent in different bat species of this genus. As pathogenicity studies have shown WCBV to be pathogenic and can lead to fatal encephalitis in animal models, with the commercially available vaccine only providing limited protection, we speculate that the effectiveness of current vaccines against MBLV will be limited. Further investigation into the effectiveness of the available biologicals is needed protection (Hanlon et al., 2005).

The question of "why have we only detected this novel virus now?" has to be asked. The lack of active and ongoing surveillance studies for lyssaviruses present in southern Africa, as well as globally, is a cause of concern and the identification of this new lyssavirus, at two separate locations, highlights the need for such programs. The discovery of this genetically diverse virus also proves the need for the development and constant improvement of the diagnostic tools able to detect the diversity of lyssavirus species. The identification of both MBLV and DUVV does provide clear evidence of the presence of known and possible novel lyssavirus species present in South Africa's insectivorous bat populations. Although the infection rate observed in this study was low, this is not uncommon for lyssaviruses (Markotter and Coertse, 2018). With the identification of three lyssavirus infections, as well as the increase in potential host range knowledge, this project has provided more information on the presence of rabies-related lyssaviruses in insectivorous bats in South Africa.

References

Acr 2019. African Chiroptera Report 2019. *African bats, Pretoria*.

Afonso, C. L., Amarasinghe, G. K., Bányai, K., Bào, Y., Basler, C. F., Bavari, S., Bejerman, N., Blasdell, K. R., Briand, F.-X. & Briese, T. 2016. Taxonomy of the order Mononegavirales: update 2016. *Archives of Virology*, 161, 2351-2360.

Albertini, A., Schoehn, G., Weissenhorn, W. & Ruigrok, R. 2008. Structural aspects of rabies virus replication. *Cellular and Molecular Life Sciences*, 65, 282-294.

Albertini, A. A., Ruigrok, R. W. & Blondel, D. 2011. Rabies virus transcription and replication. *Advances in Virus Research*. Elsevier.

Allworth, A., Murray, K. & Morgan, J. 1996. A human case of encephalitis due to a lyssavirus recently identified in fruit bats. *Communicable Diseases Intelligence*, 20, 504-504.

Amarasinghe, G. K., Ayllón, M. A., Bào, Y., Basler, C. F., Bavari, S., Blasdell, K. R., Briese, T., Brown, P. A., Bukreyev, A. & Balkema-Buschmann, A. 2019. Taxonomy of the order Mononegavirales: update 2019. *Archives of Virology*, 164, 1967-1980.

Annand, E. & Reid, P. 2014. Clinical review of two fatal equine cases of infection with the insectivorous bat strain of Australian bat lyssavirus. *Australian Veterinary Journal*, 92, 324-332.

Appleton, B., Mckenzie, J. & Christidis, L. 2004. Molecular systematics and biogeography of the bent-wing bat complex *Miniopterus schreibersii*. *Molecular Phylogenetics and Evolution*, 31, 431-439.

Arguin, P. M., Murray-Lillibridge, K., Miranda, M. E., Smith, J. S., Calaor, A. B. & Rupprecht, C. E. 2002. Serologic evidence of Lyssavirus infections among bats, the Philippines. *Emerging Infectious Diseases*, 8, 258.

Badrane, H., Bahloul, C., Perrin, P. & Tordo, N. 2001. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. *Journal of Virology*, 75, 3268-3276.

Badrane, H. & Tordo, N. 2001. Host switching in Lyssavirus history from the Chiroptera to the Carnivora orders. *Journal of Virology*, 75, 8096-8104.

Baker, M., Schountz, T. & Wang, L. F. 2013. Antiviral immune responses of bats: a review. *Zoonoses and Public Health*, 60, 104-116.

Banyard, A. C., Evans, J. S., Luo, T. R. & Fooks, A. R. 2014. Lyssaviruses and bats: emergence and zoonotic threat. *Viruses*, 6, 2974-2990.

Banyard, A. C. & Fooks, A. R. 2017. The impact of novel lyssavirus discovery. *Microbiology Australia*, 38, 17-21.

Banyard, A. C., Hayman, D., Johnson, N., Mcelhinney, L. & Fooks, A. R. 2011. Bats and lyssaviruses. *Advances in Virus Research*. Elsevier.

Barba, M. & Hadidi, A. 2017. Application of next-generation sequencing technologies to viroids. *Viroids and Satellites*. Elsevier.

Barik, S., Rud, E. W., Luk, D., Banerie, A. K. & Kang, C. Y. 1990. Nucleotide sequence analysis of the L gene of vesicular stomatitis virus (New Jersey serotype): identification of conserved domains in L proteins of non-segmented negative-strand RNA viruses. *Virology*, 175, 332-337.

Benmansour, A., Leblois, H., Coulon, P., Tuffereau, C., Gaudin, Y., Flamand, A. & Lafay, F. 1991. Antigenicity of rabies virus glycoprotein. *Journal of Virology*, 65, 4198-4203.

Blackwood, J. C., Streicker, D. G., Altizer, S. & Rohani, P. 2013. Resolving the roles of immunity, pathogenesis, and immigration for rabies persistence in vampire bats. *Proceedings of the National Academy of Sciences*, 110, 20837-20842.

Boland, T. A., Mcguone, D., Jindal, J., Rocha, M., Cumming, M., Rupprecht, C. E., Barbosa, T. F. S., De Novaes Oliveira, R., Chu, C. J. & Cole, A. J. 2014. Phylogenetic and epidemiologic evidence of multiyear incubation in human rabies. *Annals of Neurology*, 75, 155-160.

Botvinkin, A. D., Poleschuk, E. M., Kuzmin, I. V., Borisova, T. I., Gazaryan, S. V., Yager, P. & Rupprecht, C. E. 2003. Novel lyssaviruses isolated from bats in Russia. *Emerging Infectious Diseases*, 9, 1623.

Boulger, L. & Porterfield, J. 1958. Isolation of a virus from Nigerian fruit bats. Transactions of the Royal *Society of Tropical Medicine and Hygiene*, 52, 421-424.

Bourhy, H., Kissi, B., Lafon, M., Sacramento, D. & Tordo, N. 1992. Antigenic and molecular characterization of bat rabies virus in Europe. *Journal of Clinical Microbiology*, 30, 2419-2426.

Bourhy, H., Kissi, B. & Tordo, N. 1993. Molecular diversity of the Lyssavirus genus. *Virology*, 194, 70-81.

Bourhy, H., Rollin, P. E., Vincent, J. & Sureau, P. 1989. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunodiagnosis for rapid laboratory diagnosis of rabies. *Journal of Clinical Microbiology*, 27, 519-523.

Bracho, M., Moya, A. & Barrio, E. 1998. Contribution of Taq polymerase-induced errors to the estimation of RNA virus diversity. *Journal of General Virology*, 79, 2921-2928.

Ceballos, N. A., Morón, S. V., Berciano, J. M., Nicolás, O., López, C. A., Juste, J., Nevado, C. R., Setién, Á. A. & Echevarría, J. E. 2013. Novel lyssavirus in bat, Spain. *Emerging Infectious Diseases*, 19, 793.

Chaitanya, K. 2019. Structure and Organization of Virus Genomes. Genome and Genomics. Springer.

Charlton, K., Nadin-Davis, S., Casey, G. & Wandeler, A. 1997. The long incubation period in rabies: delayed progression of infection in muscle at the site of exposure. *Acta neuropathologica*, 94, 73-77.

Coertse, J., Markotter, W. & Nel, L. H. 2014. Demonstration of African Lyssavirus RNA with Real-Time Polymerase Chain Reaction. Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention. Elsevier.

Coertse, J., Weyer, J., Nel, L. H. & Markotter, W. 2010. Improved PCR methods for detection of African rabies and rabies-related lyssaviruses. *Journal of Clinical Microbiology*, 48, 3949-3955.

Coertse, J., Weyer, J., Nel, L. H. & Markotter, W. 2019. Reverse transcription recombinase polymerase amplification assay for rapid detection of canine associated rabies virus in Africa. *PloS One*, 14, e0219292.

Coetzer, A., Sabeta, C. T., Markotter, W., Rupprecht, C. E. & Nel, L. H. 2014. Comparison of biotinylated monoclonal and polyclonal antibodies in an evaluation of a direct rapid immunohistochemical test for the routine diagnosis of rabies in southern Africa. *PLoS Neglected Tropical Diseases,* 8**,** e3189.

Coetzer, J., Thomson, G. & Tustin, R. 1995. Infectious Diseases of Livestock with special reference to southern Africa. *Journal of the South African Veterinary Association,* 66**,** 106

Constantine, D. G. 1967. Bat rabies in the southwestern United States. *Public Health Reports*, 82, 867.

Corona, T. F., Böger, B., Rocha, T. C. D., Svoboda, W. K. & Gomes, E. C. 2018. Comparative analysis of Mouse Inoculation Test and Virus Isolation in Cell Culture for rabies diagnosis in animals of Parana, Brazil. *Journal of the Brazilian Society of Tropical Medicine*, 51, 39-43.

Coulon, P., Ternaux, J.-P., Flamand, A. & Tuffereau, C. 1998. An avirulent mutant of rabies virus is unable to infect motoneurons in vivo and in vitro. *Journal of Virology*, 72, 273-278.

Coxon, C., Pacey, A., Gauntlett, F. & Holland, S. 2020. Rabies in a cat in Italy [Online]. Department for Environment, Food and Rural Affairs. Available: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/897070/rabies-cat-italy-poa.pdf [Accessed 30/10/2020 2020].

Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772-772.

David, D., Hughes, G. J., Yakobson, B. A., Davidson, I., Un, H., Aylan, O., Kuzmin, I. V. & Rupprecht, C. E. 2007. Identification of novel canine rabies virus clades in the Middle East and North Africa. *Journal of General Virology*, 88, 967-980.

Dean, D. J., Abelseth M. K. & P., A. 1996. The fluorescent antibody test, Fourth Edition.

Delmas, O., Holmes, E. C., Talbi, C., Larrous, F., Dacheux, L., Bouchier, C. & Bourhy, H. 2008. Genomic diversity and evolution of the lyssaviruses. *PloS One*, 3, e2057.

Dietzgen, R. G. & Kuzmin, I. V. 2012. Rhabdoviruses: Molecular Taxonomy, Evolution, Genomics, Ecology, Host-vector Interactions, Cytopathology, and Control, Horizon Scientific Press.

Dietzschold, B., Wunner, W. H., Wiktor, T. J., Lopes, A. D., Lafon, M., Smith, C. L. & Koprowski, H. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proceedings of the National Academy of Sciences*, 80, 70-74.

Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29, 1969-1973.

Eggerbauer, E., Pfaff, F., Finke, S., Höper, D., Beer, M., Mettenleiter, T. C., Nolden, T., Teifke, J.-P., Müller, T. & Freuling, C. M. 2017. Comparative analysis of European bat lyssavirus 1 pathogenicity in the mouse model. *PLOS Neglected Tropical Diseases*, 11, 5668.

Faber, M., Faber, M.-L., Papaneri, A., Bette, M., Weihe, E., Dietzschold, B. & Schnell, M. J. 2005. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. *Journal of virology*, 79, 14141-14148.

Faber, M., Li, J., Kean, R. B., Hooper, D. C., Alugupalli, K. R. & Dietzschold, B. 2009. Effective preexposure and postexposure prophylaxis of rabies with a highly attenuated recombinant rabies virus. *Proceedings of the National Academy of Sciences*, 106, 11300-11305.

Familusi, J., Osunkoya, B., Moore, D., Kemp, G. & Fabiyi, A. 1972. A fatal human infection with Mokola virus. *The American Journal of Tropical Medicine and Hygiene*, 21, 959-963.

Fischer, M., Wernike, K., Freuling, C. M., Müller, T., Aylan, O., Brochier, B., Cliquet, F., Vázquez-Morón, S., Hostnik, P. & Huovilainen, A. 2013. A step forward in molecular diagnostics of lyssaviruses–results of a ring trial among European laboratories. *PloS One*, 8, e58372.

Fisher, C. R., Lowe, D. E., Smith, T. G., Yang, Y., Hutson, C. L., Wirblich, C., Cingolani, G. & Schnell, M. J. 2020. Lyssavirus Vaccine with a Chimeric Glycoprotein Protects across Phylogroups. *Cell Reports*, 32, 107920.

Foggin, C. 1983. Mokola virus infection in cats and a dog in Zimbabwe. *The Veterinary Record*, 113, 115.

Foggin, C. 1988. Rabies and rabies-related viruses in Zimbabwe: historical, virological and ecological aspects. CGSpace.

Foley, N. M., Goodman, S. M., Whelan, C. V., Puechmaille, S. J. & Teeling, E. 2017. Towards navigating the Minotaur's labyrinth: cryptic diversity and taxonomic revision within the speciose genus *Hipposideros* (*Hipposideridae*). *Acta Chiropterologica*, 19, 1-18.

Folmer, O. V., R 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294-299.

Fooks, A., Brookes, S., Johnson, N., Mcelhinney, L. & Hutson, A. 2003. European bat lyssaviruses: an emerging zoonosis. *Epidemiology & Infection*, 131, 1029-1039.

Francis, J. R., Nourse, C., Vaska, V. L., Calvert, S., Northill, J. A., Mccall, B. & Mattke, A. C. 2014. Australian bat lyssavirus in a child: the first reported case. *Pediatrics*, 133, e1063-e1067.

Freuling, C., Vos, A., Johnson, N., Kaipf, I., Denzinger, A., Neubert, L., Mansfield, K., Hicks, D., Nunez, A. & Tordo, N. 2009. Experimental infection of serotine bats (*Eptesicus serotinus*) with European bat lyssavirus type 1a. *Journal of General Virology*, 90, 2493-2502.

Freuling, C. M., Abendroth, B., Beer, M., Fischer, M., Hanke, D., Hoffmann, B., Höper, D., Just, F., Mettenleiter, T. C. & Schatz, J. 2013. Molecular diagnostics for the detection of Bokeloh bat lyssavirus in a bat from Bavaria, Germany. *Virus Research*, 177, 201-204.

Freuling, C. M., Beer, M., Conraths, F. J., Finke, S., Hoffmann, B., Keller, B., Kliemt, J., Mettenleiter, T. C., Mühlbach, E. & Teifke, J. P. 2011. Novel lyssavirus in Natterer's bat, Germany. *Emerging Infectious Diseases*, 17, 1519.

Freuling, C. M., Binger, T., Beer, M., Adu-Sarkodie, Y., Schatz, J., Fischer, M., Hanke, D., Hoffmann, B., Höper, D. & Mettenleiter, T. C. 2015. Lagos bat virus transmission in an *Eidolon helvum* bat colony, Ghana. *Virus Research*, 210, 42-45.

Glenn, T. C. 2011. Field guide to next-generation DNA sequencers. *Molecular ecology resources*, 11, 759-769.

Gigante, C. M., Dettinger, L., Powell, J. W., Seiders, M., Condori, R. E. C., Griesser, R., Okogi, K., Carlos, M., Pesko, K. & Breckenridge, M. 2018. Multi-site evaluation of the LN34 pan-lyssavirus real-time RT-PCR assay for post-mortem rabies diagnostics. *PloS One*, 13, e0197074.

Goldwasser, R., Kissling, R., Carski, T. & Hosty, T. 1959. Fluorescent antibody staining of rabies virus antigens in the salivary glands of rabid animals. *Bulletin of the World Health Organization*, 20, 579.

Goodwin, S., Mcpherson, J. D. & Mccombie, W. R. 2016. Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews Genetics*, 17, 333.

Görzer, I., Guelly, C., Trajanoski, S. & Puchhammer-Stöckl, E. 2010. The impact of PCR-generated recombination on diversity estimation of mixed viral populations by deep sequencing. *Journal of virological methods*, 169, 248-252.

Gunawardena, P. S., Marston, D. A., Ellis, R. J., Wise, E. L., Karawita, A. C., Breed, A. C., Mcelhinney, L. M., Johnson, N., Banyard, A. C. & Fooks, A. R. 2016. Lyssavirus in Indian Flying Foxes, Sri Lanka. *Emerging Infectious Diseases*, 22, 1456.

Guyatt, K. J., Twin, J., Davis, P., Holmes, E. C., Smith, G. A., Smith, I. L., Mackenzie, J. S. & Young, P. L. 2003. A molecular epidemiological study of Australian bat lyssavirus. *Journal of General Virology*, 84, 485-496.

Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series, 1999. [London]: Information Retrieval Ltd., c1979-c2000., 95-98.

Hampson, K., Coudeville, L., Lembo, T., Sambo, M., Kieffer, A., Attlan, M., Barrat, J., Blanton, J. D., Briggs, D. J. & Cleaveland, S. 2015. Estimating the global burden of endemic canine rabies. *PLoS Neglected Tropical Diseases*, 9, e0003709.

Hanlon, C. A., Childs, J. E. & Nettles, V. F. 1999. Recommendations of a national working group on prevention and control of rabies in the United States. Article III: Rabies in wildlife. National Working Group on Rabies Prevention and Control.

Hanlon, C. A., Kuzmin, I. V., Blanton, J. D., Weldon, W. C., Manangan, J. S. & Rupprecht, C. E. 2005. Efficacy of rabies biologics against new lyssaviruses from Eurasia. *Virus Research*, 111, 44-54.

Hanna, J. N., Carney, I. K., Smith, G. A., Deverill, J. E., Botha, J. A., Serafin, I. L., Harrower, B. J., Fitzpatrick, P. F. & Searle, J. W. 2000. Australian bat lyssavirus infection: a second human case, with a long incubation period. *The Medical Journal of Australia*, 172, 597-599.

Hayman, D. T., Fooks, A. R., Marston, D. A. & Garcia-R, J. C. 2016. The global phylogeography of lyssaviruses-challenging the'out of Africa'hypothesis. *PLoS Neglected Tropical Diseases*, 10, e0005266.

Hes, L. & Mills, G. 1997. The complete book of southern African mammals, Struik Publishers.

Hooper, P., Lunt, R., Gould, A., Samaratunga, H., Hyatt, A., Gleeson, L., Rodwell, B., Rupprecht, C., Smith, J. & Murray, P. 1997. A new lyssavirus—fhe first endemic rabies-related virus recognized in Australia. *Bulletin de l'Institut Pasteur*, 95, 209-218.

Horton, D. L., Banyard, A. C., Marston, D. A., Wise, E., Selden, D., Nunez, A., Hicks, D., Lembo, T., Cleaveland, S. & Peel, A. J. 2014. Antigenic and genetic characterization of a divergent African virus, Ikoma lyssavirus. *Journal of General Virology*, 95, 1025-1032.

Hu, S.-C., Hsu, C.-L., Lee, M.-S., Tu, Y.-C., Chang, J.-C., Wu, C.-H., Lee, S.-H., Ting, L.-J., Tsai, K.-R. & Cheng, M.-C. 2018. Lyssavirus in japanese pipistrelle, Taiwan. *Emerging Infectious Diseases*, 24, 782.

Hutcheon, J. M. & Kirsch, J. A. 2006. A moveable face: deconstructing the Microchiroptera and a new classification of extant bats. *Acta Chiropterologica*, 8, 1-10.

Ictv. 2020. ICTV virus taxonomy profile: Rhabdoviridae [Online]. Available: https://talk.ictvonline.org/ictv-reports/ictv_online_report/negative-sense-rna-viruses/w/rhabdoviridae/795/genus-lyssavirus [Accessed 2021].

Ictv 2011. 9th Report of the International Committee on Taxonomy of Viruses.(2011). Editors. King, A., Adams, M., Carstens, E., Lefkowitz, E. Elsevier Academic Press, San Diego, CA.

Ito, H., Minamoto, N., Watanabe, T., Goto, H., Rong, L. T., Sugiyama, M., Kinjo, T., Mannen, K., Mifune, K. & Konobe, T. 1994. A unique mutation of glycoprotein gene of the attenuated RC-HL strain of rabies virus, a seed virus used for production of animal vaccine in Japan. *Microbiology and Immunology*, 38, 479-482.

Jacobsen, N. & Duplessis, E. 1976. Observations on ecology and biology of cape fruit bat rousettus-aegyptiacus-leachi in eastern transvaal. *South African Journal of Science*, 72, 270-273.

Jespersen, N. E., Leyrat, C., Gérard, F. C., Bourhis, J.-M., Blondel, D., Jamin, M. & Barbar, E. 2019. The LC8-RavP ensemble structure evinces a role for LC8 in regulating lyssavirus polymerase functionality. *Journal of Molecular Biology*, 431, 4959-4977.

Johnson, N., Vos, A., Freuling, C., Tordo, N., Fooks, A. & Müller, T. 2010. Human rabies due to lyssavirus infection of bat origin. *Veterinary Microbiology*, 142, 151-159.

Johnson, N., Vos, A., Neubert, L., Freuling, C., Mansfield, K. L., Kaipf, I., Denzinger, A., Hicks, D., Nunez, A. & Franka, R. 2008. Experimental study of European bat lyssavirus type-2 infection in Daubenton's bats (Myotis daubentonii). *Journal of General Virology*, 89, 2662-2672.

Kearney, T., Keith, M., Markotter, W., Pretorius, M. & Seamark, E. 2019. Bat species (Mammalia: Chiroptera) occurring at Telperion Nature Reserve. Annals of the Ditsong National Museum of Natural History, 8, 30-42.

Kearney, T. & Taylor, P. 2011. Selection of cranial and mandible measurements for traditional morphometric analyses of southern African vesper bats of the genera *Eptesicus, Hypsugo, Neoromicia*, and *Pipistrellus* (Mammalia: Chiroptera: Vespertilionidae*). Annals of the Ditsong National Museum of Natural History*, 1, 53-61.

Kearney, T. C., Keith, M. & Seamark, E. C. 2017. New records of bat species using Gatkop Cave in the maternal season. *Mammalia*, 81, 41-48.

Kearney, T. C., Volleth, M., Contrafatto, G. & Taylor, P. J. 2002. Systematic implications of chromosome GTG-band and bacula morphology for southern African *Eptesicus* and *Pipistrellus* and several other species of *Vespertilioninae* (Chiroptera: Vespertilionidae). *Acta Chiropterologica*, 4, 55-76.

Kemp, G. E., Causey, O. R., Moore, D. L., Odelola, A. & Fabiyi, A. 1972. Mokola Virus. *The American Journal of Tropical Medicine and Hygiene*, 21, 356-359.

Kgaladi, J., Faber, M., Dietzschold, B., Nel, L. H. & Markotter, W. 2017. Pathogenicity and immunogenicity of recombinant rabies viruses expressing the Lagos bat virus matrix and glycoprotein: Perspectives for a pan-lyssavirus vaccine. *Tropical Medicine and Infectious Disease*, 2, 37.

Kgaladi, J., Wright, N., Coertse, J., Markotter, W., Marston, D., Fooks, A. R., Freuling, C. M., Müller, T. F., Sabeta, C. T. & Nel, L. H. 2013. Diversity and epidemiology of Mokola virus. *PLoS Neglected Tropical Diseases*, 7, e2511.

Klasse, P. 2014. Neutralization of virus infectivity by antibodies: old problems in new perspectives. *Advances in Biology*, 2014.

Koraka, P., Martina, B. E., Roose, J. M., Van Thiel, P.-P. A., Van Amerongen, G., Kuiken, T. & Osterhaus, A. D. 2012. In vitro and in vivo isolation and characterization of Duvenhage virus. *PLoS Pathology*, 8, e1002682.

Kuhn, J. H., Adkins, S., Alioto, D., Alkhovsky, S. V., Amarasinghe, G. K., Anthony, S. J., Avšič-Županc, T., Ayllón, M. A., Bahl, J. & Balkema-Buschmann, A. 2020. 2020 taxonomic

update for phylum *Negarnaviricota* (Riboviria: Orthornavirae), including the large orders *Bunyavirales* and Mononegavirales. *Archives of Virology*, 1-50.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547-1549.

Kuzmin, I. & Rupprecht, C. 2015. Bats and viruses: a new frontier of emerging infectious diseases. Bats and Viruses: A New Frontier of Emerging Infectious Diseases. John Wiley & Sons Inc.

Kuzmin, I. V., Hughes, G. J., Botvinkin, A. D., Orciari, L. A. & Rupprecht, C. E. 2005. Phylogenetic relationships of Irkut and West Caucasian bat viruses within the Lyssavirus genus and suggested quantitative criteria based on the N gene sequence for lyssavirus genotype definition. *Virus Research*, 111, 28-43.

Kuzmin, I. V., Mayer, A. E., Niezgoda, M., Markotter, W., Agwanda, B., Breiman, R. F. & Rupprecht, C. E. 2010. Shimoni bat virus, a new representative of the Lyssavirus genus. *Virus Research*, 149, 197-210.

Kuzmin, I. V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W., Beagley, J. C., Urazova, O. Y., Breiman, R. F. & Rupprecht, C. E. 2008a. Lagos bat virus in Kenya. *Journal of Clinical Microbiology*, 46, 1451-1461.

Kuzmin, I. V., Niezgoda, M., Franka, R., Agwanda, B., Markotter, W., Beagley, J. C., Urazova, O. Y., Breiman, R. F. & Rupprecht, C. E. 2008b. Possible emergence of West Caucasian bat virus in Africa. *Emerging Infectious Diseases*, 14, 1887.

Kuzmin, I. V., Orciari, L. A., Arai, Y. T., Smith, J. S., Hanlon, C. A., Kameoka, Y. & Rupprecht, C. E. 2003. Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationships according to N, P and G gene sequences. *Virus Research*, 97, 65-79.

Kuzmin, I. V., Schwarz, T. M., Ilinykh, P. A., Jordan, I., Ksiazek, T. G., Sachidanandam, R., Basler, C. F. & Bukreyev, A. 2017. Innate immune responses of bat and human cells to filoviruses: commonalities and distinctions. *Journal of virology*, 91.

Kuzmin, I. V., Turmelle, A. S., Agwanda, B., Markotter, W., Niezgoda, M., Breiman, R. F. & Rupprecht, C. E. 2011. Commerson's leaf-nosed bat (Hipposideros commersoni) is the likely reservoir of Shimoni bat virus. *Vector-Borne and Zoonotic Diseases*, 11, 1465-1470.

Kuzmin, I. V., Wu, X., Tordo, N. & Rupprecht, C. E. 2008c. Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. *Virus Research*, 136, 81-90.

Lafay, F., Benmansour, A., Chebli, K. & Flamand, A. 1996. Immunodominant epitopes defined by a yeast-expressed library of random fragments of the rabies virus glycoprotein map outside major antigenic sites. *Journal of General Virology*, 77, 339-346.

Lafon, M., Wiktor, T. J. & Macfarlan, R. I. 1983. Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. *Journal of General Virology*, 64, 843-851.

Langevin, C., Jaaro, H., Bressanelli, S., Fainzilber, M. & Tuffereau, C. 2002. Rabies virus glycoprotein (RVG) is a trimeric ligand for the N-terminal cysteine-rich domain of the mammalian p75 neurotrophin receptor. *Journal of Biological Chemistry*, 277, 37655-37662.

Lentz, T. L., Wilson, P. T., Hawrot, E. & Speicher, D. W. 1984. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. *Science*, 226, 847-848.

Leonova, G., Belikov, S., Kondratov, I., Krylova, N., Pavlenko, E., Romanova, E., Chentsova, I. & Petukhova, S. 2009. A fatal case of bat lyssavirus infection in Primorye Territory of the Russian Far East. *Rabies Bulletin Europe*, 33, 5-8.

Liu, Y., Zhang, S., Zhao, J., Zhang, F. & Hu, R. 2013. Isolation of Irkut virus from a Murina leucogaster bat in China. *PLoS Neglected Tropical Diseases*, 7, e2097.

Lo, K. W.-H., Naisbitt, S., Fan, J.-S., Sheng, M. & Zhang, M. 2001. The 8-kDa dynein light chain binds to its targets via a conserved (K/R) XTQT motif. *Journal of Biological Chemistry*, 276, 14059-14066.

Luis, A. D., O'shea, T. J., Hayman, D. T., Wood, J. L., Cunningham, A. A., Gilbert, A. T., Mills, J. N. & Webb, C. T. 2015. Network analysis of host–virus communities in bats and rodents reveals determinants of cross-species transmission. *Ecology letters*, 18, 1153-1162.

Lumio, J., Hillbom, M., Roine, R., Ketonen, L., Haltia, M., Valle, M., Neuvonen, E. & Lahdevirta, J. 1986. Human rabies of bat origin in Europe. *The Lancet*, 327, 378.

Mansfield, K., Johnson, N. & Fooks, A. 2004. Identification of a conserved linear epitope at the N terminus of the rabies virus glycoprotein. *Journal of General Virology*, 85, 3279-3283.

Mantari, C. T., Berrocal, A. H., Espinoza-Culupú, A. & López-Ingunza, R. 2019. Molecular characterization of the rabies virus' nucleoprotein in dogs from Arequipa, Peru. *Peruvian Journal of Experimental Medicine and Public Health*, 36, 46-53.

Markotter, W. & Coertse, J. 2018. Bat lyssaviruses, *OIE Scientific and Technical Review*.

Markotter, W., Coertse, J., De Vries, L., Geldenhuys, M. & Mortlock, M. 2020. Bat-borne viruses in Africa: a critical review. *Journal of Zoology*.

Markotter, W., Kuzmin, I., Rupprecht, C. & Nel, L. 2008a. Phylogeny of Lagos bat virus: challenges for lyssavirus taxonomy. *Virus Research*, 135, 10-21.

Markotter, W., Kuzmin, I., Rupprecht, C. E., Randles, J., Sabeta, C. T., Wandeler, A. I. & Nel, L. H. 2006. Isolation of Lagos bat virus from water mongoose. *Emerging Infectious Diseases*, 12, 1913.

Markotter, W., Kuzmin, I. V., Rupprecht, C. E. & Nel, L. H. 2009a. Lagos bat virus virulence in mice inoculated by the peripheral route. *Epidemiology & Infection*, 137, 1155-1162.

Markotter, W., Monadjem, A. & Nel, L. H. 2013. Antibodies against Duvenhage Virus in insectivorous bats in Swaziland. *Journal of Wildlife Diseases*, 49, 1000-1003.

Markotter, W., Van, C. E., Kuzmin, I., Rupprecht, C., Paweska, J., Swanepoel, R., Fooks, A., Sabeta, C., Cliquet, F. & Nel, L. 2008b. Epidemiology and pathogenicity of African bat lyssaviruses. *Developments in Biologicals*, 131, 317-325.

Markotter, W., York, D., Sabeta, C. T., Shumba, W., Zulu, G., Le Roux, K. & Nel, L. H. 2009b. Evaluation of a rapid immunodiagnostic test kit for detection of African lyssaviruses from brain material. *Onderstepoort Journal of Veterinary Research*, 76, 257-262.

Marston, D., Mcelhinney, L., Johnson, N., Müller, T., Conzelmann, K., Tordo, N. & Fooks, A. 2007. Comparative analysis of the full genome sequence of European bat lyssavirus type 1 and type 2 with other lyssaviruses and evidence for a conserved transcription termination and polyadenylation motif in the G–L 3′ non-translated region. *Journal of General Virology*, 88, 1302-1314.

Marston, D. A., Ellis, R. J., Wise, E. L., Aréchiga-Ceballos, N., Freuling, C. M., Banyard, A. C., Mcelhinney, L. M., De Lamballerie, X., Müller, T. & Fooks, A. R. 2017. Complete genome sequence of Lleida bat Lyssavirus. *Genome Announcements*, 5, e01427-16.

Marston, D. A., Horton, D. L., Ngeleja, C., Hampson, K., Mcelhinney, L. M., Banyard, A. C., Haydon, D., Cleaveland, S., Rupprecht, C. E. & Bigambo, M. 2012. Ikoma lyssavirus, highly divergent novel lyssavirus in an African civet. *Emerging Infectious Diseases*, 18, 664.

Marston, D. A., Jennings, D. L., Maclaren, N. C., Dorey-Robinson, D., Fooks, A. R., Banyard, A. C. & Mcelhinney, L. M. 2019. Pan-lyssavirus Real Time RT-PCR for Rabies Diagnosis. *Journal of Visualized Experiments*, e59709.

Marston, D. A., Mcelhinney, L. M., Ellis, R. J., Horton, D. L., Wise, E. L., Leech, S. L., David, D., De Lamballerie, X. & Fooks, A. R. 2013. Next generation sequencing of viral RNA genomes. *BMC genomics*, 14, 444.

Marston, D. A., Mcelhinney, L. M., Wise, E., Ellis, R. J., Freuling, C. M., Müller, T. F. & Fooks, A. R. 2014. Next Generation Sequencing of Lyssaviruses. Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention. Elsevier.

Mccoll, K., Tordo, N. & Aguilar, S. A. 2000. Bat lyssavirus infections. The Scientific and Technical Review OIE, 19, 177.

Mcculloch, S. 2013. Barcoding of South African bat species and evaluation of their natural exposure to lyssaviruses. MSc Dissertation, University of Pretoria, Pretoria, South Africa.

Mcelhinney, L. M., Marston, D. A., Wise, E. L., Freuling, C. M., Bourhy, H., Zanoni, R., Moldal, T., Kooi, E. A., Neubauer-Juric, A. & Nokireki, T. 2018. Molecular epidemiology and evolution of European bat lyssavirus 2. *International Journal of Molecular Sciences*, 19, 156.

Mebatsion, T., König, M. & Conzelmann, K.-K. 1996. Budding of rabies virus particles in the absence of the spike glycoprotein. *Cell*, 84, 941-951.

Mebatsion, T., Weiland, F. & Conzelmann, K.-K. 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. *Journal of Virology*, 73, 242-250.

Meester, J. 1986. Classification of southern African mammals, Transvaal Museum.

Meredith, C. & Nel, L. 1996. Further isolation of Mokola virus in South Africa. *The Veterinary Record*, 138, 119-120.

Meredith, C., Rossouw, A. & Koch, H. V. P. 1971. An unusual case of human rabies thought to be of chiropteran origin. *South African Medical Journal*, 45, 767-769.

Meslin, F., Kaplan, M. & Koprowisk, H. 2015. Laboratory techniques in rabies. Geneva: World Health Organization; 1996. & FERNANDES, MEB-Virological and serological diagnosis of rabies in bats from an urban area in the Brazilian Amazon. *Magazine of the Institute of Tropical Medicine of São Paulo*, 57, 497-503.

Miller-Butterworth, C. M., Eick, G., Jacobs, D. S., Schoeman, M. C. & Harley, E. H. 2005. Genetic and phenotypic differences between South African long-fingered bats, with a global miniopterine phylogeny. *Journal of Mammalogy*, 86, 1121-1135.

Monadjam, A. P. T., F.P.D Cotterill, M. Corrie Schoeman 2010. Bats of Southern and Central Africa, South Africa, Wits University press.

Monadjem, A., Reside, A., Cornut, J. & Perrin, M. R. 2009. Roost selection and home range of an African insectivorous bat *Nycteris thebaica* (Chiroptera, Nycteridae). *Mammalia*, 73, 353-359.

Morimoto, K., Foley, H. D., Mcgettigan, J. P., Schnell, M. J. & Dietzschold, B. 2000. Reinvestigation of the role of the rabies virus glycoprotein in viral pathogenesis using a reverse genetics approach. *Journal of Neurovirology*, 6, 373-381.

Nadin-Davis, S. A. & Real, L. A. 2011. Molecular phylogenetics of the lyssaviruses—insights from a coalescent approach. *Advances in Virus Research*. Elsevier.

Nel, L., Niezgoda, M., Hanlon, C., Morril, P., Yager, P. & Rupprecht, C. 2003. A comparison of DNA vaccines for the rabies-related virus, Mokola. *Vaccine*, 21, 2598-2606.

Nel, L., Sabeta, C., Von Teichman, B., Jaftha, J., Rupprecht, C. & Bingham, J. 2005. Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology. *Virus Research*, 109, 165-173.

Nel, L., Wandeler, A., Javangwe, S., Sabeta, C. & Bingham, J. 2001. Report of isolations of unusual lyssaviruses (rabies and Mokola virus) identified retrospectively from Zimbabwe. *Journal of the South African Veterinary Association*, 72, 92-94.

Ngoepe, E., Sabeta, C., Fehlner-Gardiner, C. & Wandeler, A. 2014. Antigenic characterisation of lyssaviruses in South Africa. *Onderstepoort Journal of Veterinary Research*, 81, 1-9.

Ni, Y., Tominaga, Y., Honda, Y., Morimoto, K., Sakamoto, S. I. & Kawai, A. 1995. Mapping and characterization of a sequential epitope on the rabies virus glycoprotein which is recognized

by a neutralizing monoclonal antibody, RG719. *Microbiology and Immunology*, 39, 693-702.

Nolden, T., Banyard, A. C., Finke, S., Fooks, A. R., Hanke, D., Höper, D., Horton, D. L., Mettenleiter, T. C., Müller, T. & Teifke, J. P. 2014. Comparative studies on the genetic, antigenic and pathogenic characteristics of Bokeloh bat lyssavirus. *The Journal of General Virology*, 95, 1647.

O'shea, T. J., Cryan, P. M., Cunningham, A. A., Fooks, A. R., Hayman, D. T., Luis, A. D., Peel, A. J., Plowright, R. K. & Wood, J. L. 2014. Bat flight and zoonotic viruses. *Emerging Infectious Diseases*, 20, 741.

Obregón-Morales, C., Aguilar-Setién, Á., Martínez, L. P., Galvez-Romero, G., Martínez-Martínez, F. O. & Aréchiga-Ceballos, N. 2017. Experimental infection of Artibeus intermedius with a vampire bat rabies virus. *Comparative Immunology, Microbiology and Infectious Diseases*, 52, 43-47.

Oie 2018. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018. OiE Paris, France.

Okada, K., Ito, N., Yamaoka, S., Masatani, T., Ebihara, H., Goto, H., Nakagawa, K., Mitake, H., Okadera, K. & Sugiyama, M. 2016. Roles of the rabies virus phosphoprotein isoforms in pathogenesis. *Journal of virology*, 90, 8226-8237.

Olival, K. J., Hosseini, P. R., Zambrana-Torrelio, C., Ross, N., Bogich, T. L. & Daszak, P. 2017. Host and viral traits predict zoonotic spillover from mammals. *Nature*, 546, 646-650.

Paweska, J. T., Van Vuren, P. J., Masumu, J., Leman, P. A., Grobbelaar, A. A., Birkhead, M., Clift, S., Swanepoel, R. & Kemp, A. 2012. Virological and serological findings in Rousettus aegyptiacus experimentally inoculated with vero cells-adapted hogan strain of Marburg virus. *PloS One*, 7, e45479.

Peel, A. J., Sargan, D. R., Baker, K. S., Hayman, D. T., Barr, J. A., Crameri, G., Suu-Ire, R., Broder, C. C., Lembo, T. & Wang, L.-F. 2013. Continent-wide panmixia of an African fruit bat facilitates transmission of potentially zoonotic viruses. *Nature communications*, 4, 1-14.

Picard-Meyer, E., Servat, A., Robardet, E., Moinet, M., Borel, C. & Cliquet, F. 2013. Isolation of Bokeloh bat lyssavirus in Myotis nattereri in France. *Archives of Virology*, 158, 2333-2340.

Picard-Meyer, E., Beven, V., Hirchaud, E., Guillaume, C., Larcher, G., Robardet, E., Servat, A., Blanchard, Y. & Cliquet, F. 2019. Lleida Bat Lyssavirus isolation in *Miniopterus schreibersii* in France. *Zoonoses and Public Health*, 66, 254-258.

Plowright, R. K., Eby, P., Hudson, P. J., Smith, I. L., Westcott, D., Bryden, W. L., Middleton, D., Reid, P. A., Mcfarlane, R. A. & Martin, G. 2015. Ecological dynamics of emerging bat virus spillover. *The Royal Society*, 20142124.

Poisson, N., Real, E., Gaudin, Y., Vaney, M.-C., King, S., Jacob, Y., Tordo, N. & Blondel, D. 2001. Molecular basis for the interaction between rabies virus phosphoprotein P and the dynein light chain LC8: dissociation of dynein-binding properties and transcriptional functionality of P. *Journal of General Virology*, 82, 2691-2696.

Pretorius, M., Broders, H., Seamark, E. & Keith, M. 2020. Climatic correlates of migrant Natal long-fingered bat (Miniopterus natalensis) phenology in north-eastern South Africa. *Wildlife Research*, 47, 404-414.

Pulmanausahakul, R., Li, J., Schnell, M. J. & Dietzschold, B. 2008. The glycoprotein and the matrix protein of rabies virus affect pathogenicity by regulating viral replication and facilitating cell-to-cell spread. *Journal of Virology*, 82, 2330-2338.

Ratnasingham, S. & Hebert, P. D. 2007. BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular Ecology Notes*, 7, 355-364.

Robinson, D. G. & Storey, J. D. 2014. subSeq: determining appropriate sequencing depth through efficient read subsampling. *Bioinformatics*, 30, 3424-3426.

Rodrigues, L. & Palmeirim, J. 2008. Migratory behaviour of the Schreiber's bat: when, where and why do cave bats migrate in a Mediterranean region? *Journal of Zoology*, 274, 116-125.

Rupprecht, C., Kuzmin, I. & Meslin, F. 2017. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. *F1000Research*, 6.

Rupprecht, C. E., Fooks, A. R., Abela-Ridder, B. & Organization, W. H. 2018. Laboratory techniques in rabies, volume 1.

Rupprecht, C. E., Turmelle, A. & Kuzmin, I. V. 2011. A perspective on lyssavirus emergence and perpetuation. *Current Opinion in Virology*, 1, 662-670.

Sabeta, C. T., Markotter, W., Mohale, D. K., Shumba, W., Wandeler, A. I. & Nel, L. H. 2007. Mokola virus in domestic mammals, South Africa. *Emerging Infectious Diseases*, 13, 1371.

Sabeta, C. T., Marston, D. A., Mcelhinney, L. M., Horton, D. L., Phahladira, B. & Fooks, A. R. 2020. Rabies in the African Civet: An Incidental Host for Lyssaviruses? *Viruses*, 12, 368.

Sacramento, D., Bourhy, H. & Tordo, N. 1991. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Molecular and Cellular Probes*, 5, 229-240.

Salk, J. J., Schmitt, M. W. & Loeb, L. A. 2018. Enhancing the accuracy of next-generation sequencing for detecting rare and subclonal mutations. *Nature Reviews Genetics*, 19, 269.

Salmon, A. B., Leonard, S., Masamsetti, V., Pierce, A., Podlutsky, A. J., Podlutskaya, N., Richardson, A., Austad, S. N. & Chaudhuri, A. R. 2009. The long lifespan of two bat species is correlated with resistance to protein oxidation and enhanced protein homeostasis. *The FASEB Journal*, 23, 2317-2326.

Saluzzo, J.-F., Rollin, P., Dauguet, C., Digoutte, J.-P., Georges, A.-J. & Sureau, P. Premier isolement du virus Mokola à partir d'un rongeur (Lophuromys sikapusi*). Annales de l'Institut Pasteur/Virologie*, 1984. Elsevier, 57-66.

Schneider, L. G. 1982. Antigenic variants of rabies virus. *Comparative Immunology, Microbiology and Infectious diseases*, 5, 101-107.

Schountz, T., Baker, M. L., Butler, J. & Munster, V. 2017. Immunological control of viral infections in bats and the emergence of viruses highly pathogenic to humans. *Frontiers in Immunology*, 8, 1098.

Schuster, S. C. 2008. Next-generation sequencing transforms today's biology. *Nature Methods*, 5, 16-18.

Shipley, R., Selden, D., Guanghui, W., Wright, E., Fooks, A. R. & Banyard, A. C. 2019a. Rabies virus: defining antigenic requirements for pan-lyssavirus neutralisation. *Access Microbiology*, 1, 165.

Shipley, R., Wright, E., Selden, D., Wu, G., Aegerter, J., Fooks, A. R. & Banyard, A. C. 2019b. Bats and viruses: Emergence of novel lyssaviruses and association of bats with viral zoonoses in the EU. *Tropical medicine and infectious disease*, 4, 31.

Shope, R. E., Murphy, F. A., Harrison, A. K., Causey, O. R., Kemp, G. E., Simpson, D. & Moore, D. L. 1970. Two African viruses serologically and morphologically related to rabies virus. *Journal of Virology*, 6, 690-692.

Simmons, N. B., Seymour, K. L., Habersetzer, J. & Gunnell, G. F. 2008. Primitive Early Eocene bat from Wyoming and the evolution of flight and echolocation. *Nature*, 451, 818-821.

Smith, J. S., Yager, P. A. & Baer, G. M. 1973. A rapid reproducible test for determining rabies neutralizing antibody. *Bulletin of the World Health Organization*, 48, 535.

Smreczak, M., Orłowska, A., Marzec, A., Trębas, P., Müller, T., Freuling, C. M. & Żmudziński, J. F. 2018. Bokeloh bat lyssavirus isolation in a Natterer's bat, Poland. *Zoonoses and Public Health*, 65, 1015-1019.

Suleiman, M. A., Kwaga, J. K., Okubanjo, O. O., Abarshi, M. M. & Kia, G. S. N. 2020. Molecular study of rabies virus in slaughtered dogs in Billiri and Kaltungo local government areas of Gombe state, Nigeria. *Acta Tropica*, 105461.

Takayama-Ito, M., Ito, N., Yamada, K., Sugiyama, M. & Minamoto, N. 2006. Multiple amino acids in the glycoprotein of rabies virus are responsible for pathogenicity in adult mice. *Virus Research*, 115, 169-175.

Taub, M. A., Bravo, H. C. & Irizarry, R. A. 2010. Overcoming bias and systematic errors in next generation sequencing data. *Genome medicine*, 2, 1-5.

Tan, G. S., Preuss, M. A., Williams, J. C. & Schnell, M. J. 2007. The dynein light chain 8 binding motif of rabies virus phosphoprotein promotes efficient viral transcription. *Proceedings of the National Academy of Sciences*, 104, 7229-7234.

Taylor, P. J. 2000. Bats of southern Africa: guide to biology, identification, and conservation, University of Kwazulu Natal Press.

Teeling, E. C., Springer, M. S., Madsen, O., Bates, P., O'brien, S. J. & Murphy, W. J. 2005. A molecular phylogeny for bats illuminates biogeography and the fossil record. *Science*, 307, 580-584.

Teng, C., Miao, F. M., Ye, L., Zhang, S. F., Zhang, F., Nan, L. & Hu, R. L. 2018. Possible transmission of irkut virus from dogs to humans. *Biomedical and environmental sciences*, 31, 146-148.

Tjørnehøj, K., Fooks, A., Agerholm, J. & Rønsholt, L. 2006. Natural and experimental infection of sheep with European bat lyssavirus type-1 of Danish bat origin. *Journal of Comparative Pathology*, 134, 190-201.

Tordo, N., Poch, O., Ermine, A., Keith, G. & Rougeon, F. 1988. Completion of the rabies virus genome sequence determination: highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. *Virology*, 165, 565-576.

Trimarchi, C., Rudd, R. & Safford Jr, M. 1996. An in vitro virus neutralization test for rabies antibody. World Health Organization, Geneva, Switzerland, 193-199.

Tuffereau, C., Bénéjean, J., Blondel, D., Kieffer, B. & Flamand, A. 1998. Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. *The EMBO journal*, 17, 7250-7259.

Van Der Merwe, M. 1982. Bats as vectors of rabies. *South African Journal of Science*, 78, 412.

Van Der Poel, W., Van Der Heide, R., Van Amerongen, G., Van Keulen, L., Wellenberg, G., Bourhy, H., Schaftenaar, W., Groen, J. & Osterhaus, A. 2000. Characterisation of a recently isolated lyssavirus in frugivorous zoo bats. *Archives of Virology*, 145, 1919-1931.

Van Thiel, P.-P. A., De Bie, R. M., Eftimov, F., Tepaske, R., Zaaijer, H. L., Van Doornum, G. J., Schutten, M., Osterhaus, A. D., Majoie, C. B. & Aronica, E. 2009. Fatal human rabies due to Duvenhage virus from a bat in Kenya: failure of treatment with coma-induction, ketamine, and antiviral drugs. *PLoS Neglected Tropical Diseases*, 3, e428.

Wadhwa, A., Wilkins, K., Gao, J., Condori, R. E. C., Gigante, C. M., Zhao, H., Ma, X., Ellison, J. A., Greenberg, L. & Velasco-Villa, A. 2017. A pan-lyssavirus Taqman real-time RT-PCR assay for the detection of highly variable rabies virus and other lyssaviruses. *PLoS neglected tropical diseases*, 11, e0005258.

Walker, P. J., Blasdell, K. R., Calisher, C. H., Dietzgen, R. G., Kondo, H., Kurath, G., Longdon, B., Stone, D. M., Tesh, R. B. & Tordo, N. 2018. ICTV virus taxonomy profile: Rhabdoviridae. *Journal of General Virology*, 99, 447-448.

Wang, L.-F., Walker, P. J. & Poon, L. L. 2011. Mass extinctions, biodiversity and mitochondrial function: are bats 'special'as reservoirs for emerging viruses? *Current Opinion in Virology*, 1, 649-657.

Wang, Y., Xu, W., Guo, H., Gong, W., He, B., Tu, Z., Tu, C. & Feng, Y. 2019. Evaluation of a Universal Nested Reverse Transcription Polymerase Chain Reaction for the Detection of Lyssaviruses. Journal of Visualized Experiments, e59428.

Weyer, J., Kuzmin, I. V., Rupprecht, C. E. & Nel, L. H. 2008. Cross-protective and cross-reactive immune responses to recombinant vaccinia viruses expressing full-length lyssavirus glycoprotein genes. *Epidemiology & Infection*, 136, 670-678.

Weyer, J., Szmyd-Potapczuk, A. V., Blumberg, L. H., Leman, P. A., Markotter, W., Swanepoel, R., Paweska, J. T. & Nel, L. H. 2011. Epidemiology of human rabies in South Africa, 1983–2007. *Virus Research*, 155, 283-290.

Whelan, S. P., Barr, J. N. & Wertz, G. W. 2000. Identification of a minimal size requirement for termination of vesicular stomatitis virus mRNA: implications for the mechanism of transcription. *Journal of virology*, 74, 8268-8276.

Willoughby Jr, R. E., Tieves, K. S., Hoffman, G. M., Ghanayem, N. S., Amlie-Lefond, C. M., Schwabe, M. J., Chusid, M. J. & Rupprecht, C. E. 2005. Survival after treatment of rabies with induction of coma. *New England Journal of Medicine*, 352, 2508-2514.

Winkler, W. G., Fashinell, T. R., Leffingwell, L., Howard, P. & Conomy, J. P. 1973. Airborne rabies transmission in a laboratory worker. *Jama*, 226, 1219-1221.

Wilson, D. E. & Reeder, D. M. 2005. Mammal species of the world: a taxonomic and geographic reference, JHU Press.

Wise, E., Marston, D., Banyard, A., Goharriz, H., Selden, D., Maclaren, N., Goddard, T., Johnson, N., Mcelhinney, L. & Brouwer, A. 2017. Passive surveillance of United Kingdom bats for lyssaviruses (2005–2015). *Epidemiology & Infection*, 145, 2445-2457.

Wong, S., Lau, S., Woo, P. & Yuen, K. Y. 2007. Bats as a continuing source of emerging infections in humans. *Reviews in Medical Virology*, 17, 67-91.

Wunner, W. & Conzelmann, K. 2013. Chapter 2: Rabies virus. Rabies–Scientific basis of the disease and its management. 3rd ed. San Diego: Elsevier Inc, 17-60.

Zhang, G., Cowled, C., Shi, Z., Huang, Z., Bishop-Lilly, K. A., Fang, X., Wynne, J. W., Xiong, Z., Baker, M. L. & Zhao, W. 2013. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. *Science*, 339, 456-460.

Zhou, P., Cowled, C., Mansell, A., Monaghan, P., Green, D., Wu, L., Shi, Z., Wang, L.-F. & Baker, M. L. 2014. IRF7 in the Australian black flying fox, Pteropus alecto: evidence for a unique expression pattern and functional conservation. *PLoS One*, 9, e103875.

Zhou, P., Cowled, C., Todd, S., Crameri, G., Virtue, E. R., Marsh, G. A., Klein, R., Shi, Z., Wang, L.-F. & Baker, M. L. 2011. Type III IFNs in pteropid bats: differential expression patterns provide evidence for distinct roles in antiviral immunity. *The Journal of Immunology*, 186, 3138-3147.

Zhou, P., Tachedjian, M., Wynne, J. W., Boyd, V., Cui, J., Smith, I., Cowled, C., Ng, J. H., Mok, L. & Michalski, W. P. 2016. Contraction of the type I IFN locus and unusual constitutive expression of IFN-α in bats. *Proceedings of the National Academy of Sciences*, 113, 2696-2701.

Appendix A

Table A1 Insectivorous bat samples tested for the presence of lyssavirus nucleic acid.

UP nr/ Museum nr	Collection date	Bat species	ID method	Province	Coordinates	SIte	Additional submission comments
UP 0004	2004 Aug 20	*Neoromicia (Laephotis) nana*	Morphological	Mpumalanga	-34.472989, 19.668606	Laughing waters farm, Nelspruit	None
UP 0005	2004 Aug 21	*Neoromicia (Laephotis) nana*	Morphological	Mpumalanga	-34.472989, 19.668606	Laughing waters farm, Nelspruit	None
UP 0006	2004 Aug 21	*Neoromicia (Laephotis) nana*	Morphological	Mpumalanga	-34.472989, 19.668606	Laughing waters farm, Nelspruit	None
UP 0012	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None
UP 0013	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None
UP 0014	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None
UP 0015	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None

UP 0016	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None
UP 0017	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None
UP 0018	2004 Aug 26	*Pipistrellus hesperidus*	Genetic	Eastern Cape	-33.069128, 26.816924	Andries Vosloo Kudu Reserve, Grahamstown	None
UP 0056, DM7842	2004 May 02	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-29.857855, 30.722425	Shongeni valley, Shongweni Nature Reserve	None
UP 0057, DM8002	2003 Feb 20	*Otomops martiensseni*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0058	2004 Apr 15	*Pipistrellus hesperidus*	Morphological	KwaZulu-Natal	-28.735800, 31.136096	Nkandla Forest Reserve, Nkandla	None
UP 0059, DM7838	2003 Nov 11	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-29.834418, 30.926404	Westville	None
UP 0060	2004 Apr 15	*Pipistrellus hesperidus*	Morphological	KwaZulu-Natal	-28.735800, 31.136096	Nkandla Forest Reserve, Nkandla	None
UP 0061, DM7906	2003 May 26	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	Not recorded	Not recorded	None
UP 0063	2004 Jun 17	*Pipistrellus hesperidus*	Morphological	KwaZulu-Natal	-30.036554, 30.896156	Amanzimtoti	None
UP 0064, DM8013	2005 Feb 23	*Pipistrellus hesperidus*	Museum voucher	KwaZulu-Natal	-27.559892, 32.664073	Sodwana Bay National Park	None

UP 0065	2004 May 15	*Rhinolophus darlingi*	Morphological	KwaZulu-Natal	-30.310606, 30.664202	Umzino	None
UP 0066, DM7910	2003 Apr 22	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-29.839963, 30.840120	Pinetown	None
UP 0067, DM7913	2002 Sep 01	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-30.115940, 30.837709	Illovo	None
UP 0068, DM8004	2004 May 19	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0070, DM7914	2004 May 27	*Otomops martiensseni*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0071, DM8382	2005 May 15	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0082, DM8376	2005 May 15	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0084, DM8373	2005 May 14	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-28.882841, 31.464582	Eshowe	None
UP 0085, DM7848	2005 Jun 24	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0098, DM8439	2005 Aug 08	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-28.764711, 31.897268	Empangeni	None
UP 0099, DM8381	2005 May 15	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0100, DM8383	2005 May 15	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0101, DM7915	2005 Jun 24	*Neoromicia (Laephotis) nana*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0102, DM8379	2005 May 15	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0103, DM8384	2005 May 15	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0104	2004 Sep 28	*Rhinolophus hildebrandtii* s.l.	Morphological	Mpumalanga	Not recorded	Not recorded	None
UP 0109, DM7886	2004 Sep 28	*Rhinolophus hildebrandtii* s.l.	Museum voucher	Mpumalanga	Not recorded	Not recorded	None
UP 0111, DM7909	2004 May 17	*Otomops martiensseni*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None

UP 0115, DM8369	2005 May 14	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-28.885552, 31.361206	Entumeni	None
UP 0116, DM7908	2003 Feb 07	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0118, DM8374	2005 May 14	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0119, DM8001	2003 Feb 07	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0124, DM8000	2003 Apr 25	*Neoromicia (Laephotis) nana*	Museum voucher	KwaZulu-Natal	-29.668920, 31.118235	Umdloti	None
UP 0125, DM8380	2005 May 15	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0126, DM8003	2004 Feb 27	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-30.036554, 30.896156	Amanzimtoti	None
UP 0127, DM7904	2002 Dec 17	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	-29.870801, 30.506680	Eston	None
UP 0129, DM8377	2005 May 15	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0131, DM8375	2005 May 14	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-28.882841, 31.464582	Eshowe	None
UP 0132, DM8440	2006 Jan 25	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0134, DM7907	2004 Jan 04	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	Not recorded	Not recorded	None
UP 0135, DM8421	2005 Sep 01	*Otomops martiensseni*	Museum voucher	KwaZulu-Natal	-29.839963, 30.840120	Pinetown	None
UP 0138, DM8378	2005 May 15	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-28.582087, 31.401064	Melmoth	None
UP 0139, DM8429	2005 Aug 01	*Miniopterus natalensis*	Museum voucher	KwaZulu-Natal	Not recorded	Not recorded	None
UP 0140, DM8419	2006 Jan 25	*Otomops martiensseni*	Museum voucher	KwaZulu-Natal	Not recorded	Not recorded	None
UP 0141, DM8420	2006 Jan 25	*Otomops martiensseni*	Museum voucher	KwaZulu-Natal	-29.851041, 31.021642	Durban	None
UP 0148	2006 Jun 23	*Otomops martiensseni*	Morphological	KwaZulu-Natal	-29.851041, 31.021642	Durban	Carcass received from The Bat Interest Group Of Kwa Zulu Natal
UP 0149	2006 May 29	*Chaerephon (Mops) pumilus*	Genetic	KwaZulu-Natal	Not recorded	Not recorded	Carcass received from The Bat Interest Group Of Kwa Zulu Natal

UP 0151	2006 Mar 25	*Scotophilus dinganii*	Genetic	KwaZulu-Natal	-29.851041, 31.021642	Durban	Carcass received from The Bat Interest Group Of Kwa Zulu Natal
UP 0152	2006 Apr 20	*Tadarida aegyptiaca*	Genetic	KwaZulu-Natal	-29.851041, 31.021642	Durban	Carcass received from The Bat Interest Group Of Kwa Zulu Natal
UP 0153	2006 Apr 10	*Chaerephon (Mops) pumilus*	Genetic	KwaZulu-Natal	-29.851041, 31.021642	Durban	Carcass received from The Bat Interest Group Of Kwa Zulu Natal
UP 0154, TM47967	2007 Jun 10	*Myotis welwitschii*	Museum voucher	Mpumalanga	-25.580473, 30.190405	Vlakfontein	None
UP 0155, TM47871	2006 Jan 11	*Myotis tricolor*	Museum voucher	Mpumalanga	-25.369997, 30.699825	Sudwala kraal	Carcass received from Mpumalanga Tourism and Parks Agency
UP 0156, TM48048	2007 Dec 16	*Tadarida aegyptiaca*	Museum voucher	North West	-27.6245, 24.63344	Taung World Heritage Site	None
UP 0157, TM48049	2007 Dec 16	*Tadarida aegyptiaca*	Museum voucher	North West	-27.6245, 24.63344	Taung World Heritage Site	None
UP 0158, TM48050	2007 Dec 16	*Tadarida aegyptiaca*	Museum voucher	North West	-27.6245, 24.63344	Taung World Heritage Site	None
UP 0159, TM48047	2007 Dec 16	*Tadarida aegyptiaca*	Museum voucher	North West	-27.6245, 24.63344	Taung World Heritage Site	None
UP 0160, TM48034	2007 Dec 14	*Rhinolophus denti*	Museum voucher	North West	-27.61543, 24.63005	Taung World Heritage Site	None
UP 0161, TM48032	2007 Dec 14	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-27.61543, 24.63005	Taung World Heritage Site	None
UP 0162, TM48036	2007 Dec 14	*Rhinolophus denti*	Museum voucher	North West	-27.61543, 24.63005	Taung World Heritage Site	None
UP 0163, TM48035	2007 Dec 14	*Rhinolophus denti*	Museum voucher	North West	-27.61543, 24.63005	Taung World Heritage Site	None
UP 0164, TM48033	2007 Dec 14	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-27.61543, 24.63005	Taung World Heritage Site	None
UP 0165, TM48042	2007 Dec 15	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-27.61376, 24.62976	Taung World Heritage Site	None
UP 0166, TM48046	2007 Dec 15	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	North West	-27.61428, 24.62896	Taung World Heritage Site	None

UP 0167, TM48043	2007 Dec 15	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-27.61428, 24.62896	Taung World Heritage Site	None
UP 0168, TM48044	2007 Dec 15	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-27.61428, 24.62896	Taung World Heritage Site	None
UP 0169, TM48045	2007 Dec 15	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-27.61428, 24.62896	Taung World Heritage Site	None
UP 0171, TM48037	2007 Dec 14	*Rhinolophus denti*	Museum voucher	North West	-27.61543, 24.63005	Taung World Heritage Site	None
UP 0172, TM48041	2007 Dec 15	*Rhinolophus denti*	Museum voucher	North West	-27.61408, 24.62941	Taung World Heritage Site	None
UP 0173, TM48038	2007 Dec 15	*Eptesicus hottentotus*	Museum voucher	North West	-27.61408, 24.62941	Taung World Heritage Site	None
UP 0174, TM48040	2007 Dec 15	*Rhinolophus damarensis*	Museum voucher	North West	-27.61408, 24.62941	Taung World Heritage Site	None
UP 0190, TM48014	2007 Dec 01	*Nycteris thebaica*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	Caught with pup attached
UP 0192, TM48016	2007 Dec 01	*Rhinolophus simulator*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0193, TM48017	2007 Dec 01	*Rhinolophus simulator*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0194, TM48018	2007 Dec 01	*Rhinolophus damarensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	Caught with pup attached
UP 0195, TM48019	2007 Dec 01	*Rhinolophus damarensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	Pup of UP0194
UP 0196, TM48020	2007 Dec 01	*Rhinolophus damarensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	Caught with pup attached
UP 0197, TM48021	2007 Dec 01	*Rhinolophus damarensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	Pup of UP0196

UP 0198, TM48022	2007 Dec 01	*Miniopterus natalensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0199, TM48023	2007 Dec 01	*Miniopterus natalensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0200, TM48024	2007 Dec 01	*Miniopterus natalensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0201, TM48025	2007 Dec 01	*Miniopterus natalensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0202, TM48026	2007 Dec 01	*Miniopterus natalensis*	Museum voucher	North West	-24.76622, 26.37098	Madikwe game reserve	None
UP 0203, TM48027	2007 Dec 01	*Scotophilus dinganii*	Museum voucher	North West	-24.76622, 26.32628	Madikwe game reserve	None
UP 0204, TM48028	2007 Dec 01	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-24.79699, 26.30092	Madikwe game reserve	None
UP 0205, TM48029	2007 Dec 01	*Sauromys petrophilus*	Museum voucher	North West	-24.79699, 26.30092	Madikwe game reserve	None
UP 0206, TM48030	2007 Dec 01	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-24.79699, 26.30092	Madikwe game reserve	None
UP 0207, TM48031	2007 Dec 01	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-24.79699, 26.30092	Madikwe game reserve	None
UP 0288	2007 Nov 07	*Miniopterus fraterculus*	Genetic	North West	-26.861075, 27.287641	Thabela thabeng	Bat found dead in cave
UP 0291	2007 Nov 07	*Miniopterus fraterculus*	Genetic	North West	-26.861075, 27.287641	Thabela thabeng	Bat found dead in cave
UP 0310	2008 Feb 24	*Neoromicia (Laephotis) capensis*	Genetic	Mpumalanga	-25.091742, 30.563794	Paardeplaats	Child bitten by bat
UP 0314	2008 Jun 10	*Chaerephon (Mops) pumilus*	Morphological	KwaZulu-Natal	-30.203816, 30.780837	Umkomaas	None
UP 0473	2007 May 10	*Mops condylurus*	Morphological	Gauteng	-26.397163, 28.463172	Nigel	None

UP 0639	2010 Sep 03	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	-25.823356, 28.084432	Hennopsrivier	Carcass received from GNORBIG
UP 0713; TM48494	2010 Feb 01	*Glauconycteris variegate*	Genetic, Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0716; TM48497	2010 Feb 01	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0717; TM48498	2010 Feb 01	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0719; TM48500	2010 Feb 01	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0720; TM48501	2010 Feb 01	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0721; TM48502	2010 Feb 01	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0722; TM48503	2010 Feb 01	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0723; TM48504	2010 Feb 01	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0724; TM48505	2010 Feb 01	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0725; TM48506	2010 Feb 01	*Neoromicia (Laephotis) zuluensis*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0726; TM48507	2010 Feb 01	*Myotis tricolor*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0728; TM48509	2010 Feb 01	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0733; TM48512	2010 Feb 02	*Mops condylurus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	Pregnant
UP 0734; TM48513	2010 Feb 02	*Mops condylurus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None

UP 0739; TM48518	2010 Feb 02	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0741; TM48502	2010 Feb 02	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0745; TM48523	2010 Feb 02	*Pipistrellus rusticus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0746; TM48524	2010 Feb 02	*Pipistrellus rusticus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0747; TM48525	2010 Feb 02	*Pipistrellus rusticus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0748; TM48526	2010 Feb 02	*Pipistrellus rusticus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0749; TM48527	2010 Feb 02	*Pipistrellus rusticus*	Museum voucher	Limpopo	-22.42601, 31.2987	Kruger National Park	None
UP 0755; TM48528	2010 Feb 03	*Taphozous mauritianus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0758; TM48531	2010 Feb 03	*Chaerephon (Mops) pumilus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0759; TM48532	2010 Feb 03	*Chaerephon (Mops) pumilus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0761; TM48534	2010 Feb 03	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0762; TM48535	2010 Feb 03	*Neoromicia (Laephotis)* cf. *helios*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0763; TM48536	2010 Feb 03	*Neoromicia (Laephotis) cf. helios*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0764; TM48537	2010 Feb 03	*Neoromicia (Laephotis) cf. helios*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None

UP 0766; TM48539	2010 Feb 03	*Scotophilus dinganii*	Genetic, Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0767; TM48540	2010 Feb 03	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0768; TM48541	2010 Feb 03	*Mops condylurus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0769; TM48542	2010 Feb 03	*Taphozous mauritianus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0770; TM48543	2010 Feb 03	*Mops condylurus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0771; TM48544	2010 Feb 03	*Mops condylurus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0772; TM48545	2010 Feb 03	*Chaerephon (Mops) ansorgei*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0774; TM48547	2010 Feb 03	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0775; TM48548	2010 Feb 03	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0779; TM48552	2010 Feb 03	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0780; TM48553	2010 Feb 03	*Scotophilus leucogaster*	Genetic, Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0781; TM48554	2010 Feb 03	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0782; TM48555	2010 Feb 03	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0783; TM48556	2010 Feb 03	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None

UP 0784; TM48557	2010 Feb 03	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0785; TM48558	2010 Feb 03	*Scotophilus leucogaster*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0786; TM48559	2010 Feb 03	*Scotophilus leucogaster*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0787; TM48560	2010 Feb 03	*Scotophilus leucogaster*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0827; TM48611	2010 Apr 12	*Glauconycteris variegata*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768556	Rocktail Beach Camp	None
UP 0828; TM48612	2010 Apr 14	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0829; TM48613	2010 Apr 14	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0830; TM48614	2010 Apr 13	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0831; TM48615	2010 Apr 13	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0833; TM48617	2010 Apr 13	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0834; TM48618	2010 Apr 13	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0835; TM48619	2010 Apr 13	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0836; TM48620	2010 Apr 13	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0837; TM48621	2010 Apr 13	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None

UP 0838; TM48622	2010 Apr 13	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0839; TM48623	2010 Apr 14	*Chaerephon (Mops) pumilus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0840; TM48624	2010 Apr 13	*Pipistrellus hesperidus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0842; TM48626	2010 Apr 13	*Pipistrellus hesperidus*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0843; TM48627	2010 Apr 14	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0844; TM48628	2010 Apr 14	*Kerivoula (Glauconycteris) argentata*	Museum voucher	KwaZulu-Natal	-27.221520, 32.793200	Rocktail Beach Camp	None
UP 0845; TM48629	2010 Apr 14	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.262925, 32.76855	Rocktail Beach Camp	None
UP 0846; TM48630	2010 Apr 14	*Glauconycteris variegata*	Museum voucher	KwaZulu-Natal	-27.262925, 32.76855	Rocktail Beach Camp	None
UP 0847; TM48631	2010 Apr 14	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.262925, 32.76855	Rocktail Beach Camp	None
UP 0848; TM48632	2010 Apr 14	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.262925, 32.76855	Rocktail Beach Camp	None
UP 0854; TM48635	2010 Apr 15	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-27.22152, 32.7932	Rocktail Beach Camp	None
UP 0855; TM48636	2010 Apr 15	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-27.22152, 32.7932	Rocktail Beach Camp	None
UP 0856; TM48637	2010 Apr 15	*Nycteris thebaica*	Museum voucher	KwaZulu-Natal	-27.22152, 32.7932	Rocktail Beach Camp	None
UP 0865; TM48638	2010 Apr 15	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None

UP 0866; TM48639	2010 Apr 15	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None
UP 0867; TM48640	2010 Apr 15	*Scotophilus dinganii*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None
UP 0868; TM48641	2010 Apr 15	*Glauconycteris variegata*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None
UP 0869; TM48642	2010 Apr 15	*Pipistrellus hesperidus*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None
UP 0870; TM48643	2010 Apr 15	*Pipistrellus hesperidus*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None
UP 0871; TM48644	2010 Apr 15	*Pipistrellus hesperidus*	Museum voucher	KwaZulu-Natal	-27.262925, 32.768550	Rocktail Beach Camp	None
UP 0904	2009 Jul 18	*Miniopterus fraterculus*	Genetic	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 0912; TM48567	2010 Aug 11	*Hipposideros caffer*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	Pregnant
UP 0913; TM48568	2010 Aug 11	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	Pregnant
UP 0914; TM48569	2010 Aug 11	*Nycticeinops schlieffeni*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0915; TM48570	2010 Aug 11	*Neoromicia (Laephotis) capensis*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	Pregnant
UP 0920; TM48571	2010 Sep 11	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0921; voucher in Kruger National Park collection	2010 Sep 11	*Neoromicia (Laephotis) cf. helios*	Morphological	Limpopo	-22.42151, 31.2238	Kruger National Park	Pregnant
UP 0922; TM48572	2010 Sep 11	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None

UP 0923; TM48573	2010 Sep 11	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	None
UP 0929; TM48576	2010 Oct 11	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42601, 31.29873	Kruger National Park	None
UP 0930; TM48577	2010 Oct 11	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.42601, 31.29873	Kruger National Park	None
UP 0931; TM48578	2010 Oct 11	*Neoromicia (Laephotis) helios*	Museum voucher	Limpopo	-22.42601, 31.29873	Kruger National Park	Pregnant
UP 0932; TM48579	2010 Oct 11	*Pipistrellus rusticus*	Museum voucher	Limpopo	-22.42601, 31.29873	Kruger National Park	Pregnant
UP 0934; TM48581	2010 Oct 11	*Chaerephon (Mops) pumilus*	Museum voucher	Limpopo	-22.42601, 31.29873	Kruger National Park	None
UP 0947; TM48582	2010 Nov 11	*Rhinolophus landeri*	Museum voucher	Limpopo	-22.42601, 31.29873	Kruger National Park	Pregnant
UP 0948; TM48583	2010 Nov 11	*Hipposideros caffer*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	Pregnant
UP 0949; TM48584	2010 Dec 11	*Hipposideros caffer*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0951; TM48586	2010 Dec 11	*Hipposideros caffer*	Museum voucher	Limpopo	-22.42151, 31.2238	Kruger National Park	Pregnant
UP 0952; TM48587	2010 Dec 11	*Neoromicia (Laephotis) helios*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0953; TM48588	2010 Dec 11	*Taphozous mauritianus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0954; TM48589	2010 Dec 11	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0955; TM48590	2010 Dec 11	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0956; TM48591	2010 Dec 11	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None

UP 0957; TM48592	2010 Dec 11	*Scotophilus viridis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0958; TM48593	2010 Dec 11	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Lactating
UP 0959; TM48594	2010 Dec 11	*Scotophilus dinganii*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0960; TM48595	2010 Dec 11	*Scotophilus leucogaster*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0961; TM48596	2010 Dec 11	*Neoromicia (Laephotis) zuluensis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	lactating
UP 0962; TM48597	2010 Dec 11	*Neoromicia (Laephotis) nana*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0963; TM48598	2010 Dec 11	*Neoromicia (Laephotis) capensis*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Lactating
UP 0964; TM48599	2010 Dec 11	*Neoromicia (Laephotis) rendalli*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Lactating
UP 0965; TM48600	2010 Dec 11	*Chaerephon (Mops) ansorgei*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0966; TM48601	2010 Dec 11	*Mops condylurus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 0967; TM48602	2010 Dec 11	*Mops condylurus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	Pregnant
UP 0968; TM48603	2010 Dec 11	*Mops condylurus*	Museum voucher	Limpopo	-22.34657, 31.11595	Kruger National Park	None
UP 1007; TM48653	2010 Dec 10	*Tadarida aegyptiaca*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None
UP 1008; TM48646	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None

UP 1009; TM48651	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None
UP 1010; TM48650	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None
UP 1011; TM48658	2010 Dec 10	*Pipistrellus rusticus*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1012; TM48660	2010 Dec 10	*Neoromicia (Laephotis) capensis*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None
UP 1013; TM48656	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1014; TM48654	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1015; TM48648	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1016; TM48645	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1017; TM48649	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1018; TM48659	2010 Dec 10	*Scotophilus dinganii*	Genetic, Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1019; TM48652	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None
UP 1020; TM48657	2010 Dec 10	*Scotophilus dinganii*	Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	None
UP 1022; TM48647	2010 Dec 10	*Pipistrellus rusticus*	Genetic, Museum voucher	North West	-25.73903, 27.23011	Kgaswane Nature Reserve	Lactating
UP 1052; TM48664	2010 Dec 11	*Pipistrellus rusticus*	Museum voucher	North West	-25.7202, 27.18533	Kgaswane Nature Reserve	None

UP 1054	2010 Dec 03	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	-25.736022, 28.190374	Pretoria Zoo	None
UP 1055	2010 Dec 01	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	-25.736022, 28.190374	Pretoria Zoo	None
UP 1056	2010 Dec 01	*Tadarida aegyptiaca*	Genetic	Gauteng	-25.736022, 28.190374	Pretoria Zoo	None
UP 1363	2011 Sep 13	*Scotophilus dinganii*	Morphological	Limpopo	-23.03262, 29.92965	71 Kameel street, Louis Trichardt	Bat found dead
UP 1364	2011 Sep 13	*Mops midas*	Morphological	Limpopo	-23.03262, 29.92965	71 Kameel street, Louis Trichardt	Bat found dead
UP 1366	2011 Sep 14	*Rhinolophus simulator*	Morphological	Limpopo	Not recorded	Labuschagne farm, Louis Trichardt	Bat found torpid on cave wall
UP 1367	2011 Sep 14	*Rhinolophus clivosus*	Morphological	Limpopo	Not recorded	Labuschagne farm, Louis Trichardt	None
UP 1368	2011 Sep 14	*Rhinolophus hildebrandtii s.l.*	Morphological	Limpopo	Not recorded	Labuschagne farm, Louis Trichardt	Pregnant
UP 1369	2011 Sep 24	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	-26.0147. 28.0222	FreeMe Wildlife Rehabilitation Centre	Bat suffering from hair loss
UP 1370	2010 Mar 13	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1371	2010 Mar 13	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1372	2010 Mar 13	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1373	2010 Oct 01	*Miniopterus natalensis*	Genetic	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	Bat found dead in cave
UP 1374	2011 Apr 01	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1376	2011 Apr 01	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None

UP 1378	2011 Apr 01	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1382	2011 May 06	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1383	2011 May 06	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1384	2011 May 06	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1385	2011 May 06	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1386	2011 May 06	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1387	2011 May 06	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	Bat found dead in cave
UP 1389	2011 Jun 03	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1390	2011 Jun 03	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1391	2011 Jun 03	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1392	2011 Jun 03	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1395	2011 Jun 29	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1396	2011 Jun 29	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1397	2011 Jun 29	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1398	2011 Jun 29	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1399	2011 Jun 29	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 1421	2011 Nov 27	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	Not recorded	Not recorded	None
UP 1422	2011 Dec 05	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	Not recorded	Not recorded	None
UP 1423	2011 Dec 17	*Pipistrellus rusticus*	Morphological	Gauteng	Not recorded	Not recorded	None

UP 1424	2011 Jul 25	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	Not recorded	Not recorded	Necropsy identified punctured stomach
UP 1425	2011 Jul 25	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	Not recorded	Not recorded	Necropsy identified enlarged liver
UP 1426	2011 Jul 25	*Scotophilus dinganii*	Genetic	Gauteng	Not recorded	Not recorded	None
UP 1427	2011 Jul 25	*Scotophilus dinganii*	Genetic	Gauteng	Not recorded	Not recorded	None
UP 1428	2012 Apr 21	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	-25.736022, 28.190374	Pretoria Zoo	None
UP 1485	2012 Jun 12	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1486	2012 Jun 12	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1487	2012 Jun 12	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1488	2012 Jun 12	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1489	2012 Jun 12	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1490	2012 Jun 12	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1491	2012 Jun 12	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1492	2012 Jun 12	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1493	2012 Jun 12	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1494	2012 Jun 12	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1495	2012 Jun 12	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1540	2012 Aug 19	*Nycteris thebaica*	Morphological	Limpopo	-24.777490, 27.734082	Rooiberg	None
UP 1547	2012 Sep 20	*Tadarida aegyptiaca*	Genetic	Gauteng	-25.736022, 28.190374	Pretoria Zoo	None
UP 1601	2012 Nov 08	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1604	2012 Nov 08	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None

UP 1700; NHCPHE MAM-29	2013 Jan 16	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.58943, 27.69429	Donkerpoort 448	None
UP 1702; NHCPHE MAM-45	2013 Jan 16	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58943, 27.69429	Donkerpoort 448	None
UP 1703; TM50559	2013 Jan 17	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58943, 27.69429	Donkerpoort 448	None
UP 1717; NHCPHE MAM-30	2013 Jan 18	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58943, 27.69429	Donkerpoort 448	None
UP 1718; TM50560	2013 Jan 18	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58941, 27.68473	Randstephne 455	None
UP 1719; NHCPHE MAM-31	2013 Jan 18	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58941, 27.68473	Randstephne 455	None
UP 1723; TM50561	2013 Jan 18	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58941, 27.68473	Randstephne 455	None
UP 1731; NHCPHE MAM-46	2013 Jan 18	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.58941, 27.68473	Randstephne 455	None
UP 1764; NHCPHE MAM-47	2013 Jan 20	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1786; NHCPHE MAM-32	2013 Jan 20	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1787; TM49186	2013 Jan 19	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1788; TM49187	2013 Jan 19	*Pipistrellus rusticus*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1790; TM50558	2013 Jan 20	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1791; TM50589	2013 Jan 20	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1792; TM50554	2013 Jan 20	*Scotophilus dinganii*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1793; TM50555	2013 Jan 20	*Scotophilus dinganii*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None

UP 1794; NHCPHE MAM-28	2013 Jan 20	*Scotophilus dinganii*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1795; TM50553	2013 Jan 20	*Scotophilus dinganii*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 1808; TM50551	2013 Jan 22	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 1810; TM50552	2013 Jan 22	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 1811; NHCPHE MAM-27	2013 Jan 22	*Nycteris thebaica*	Genetic, Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 1816, TM48672	2013 Feb 05	*Cloeotis percivali*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1862, TM48673	2013 Feb 05	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1863, TM48674	2013 Feb 05	*Cloeotis percivali*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1864, TM48675	2013 Feb 05	*Rhinolophus darlingi*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 1865, TM48676	2013 Feb 05	*Myotis tricolor*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2002, TM48677	2013 Mar 05	*Hipposideros caffer*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2003, TM48678	2013 Mar 05	*Hipposideros caffer*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2004, TM48679	2013 Mar 05	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2005, TM48680	2013 Mar 05	*Rhinolophus darlingi*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2006, TM48681	2013 Mar 05	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2007, TM48682	2013 Mar 05	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2008, TM48683	2013 Mar 05	*Rhinolophus darlingi*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2009, TM48684	2013 Mar 05	*Rhinolophus darlingi*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2010, TM48685	2013 Mar 05	*Hipposideros caffer*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None

UP 2011, TM48686	2013 Mar 05	*Hipposideros caffer*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 2766, TM50659	2013 Jul 02	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Bat was old, bloody and with swollen stomach and pale liver
UP 3012, TM50645	2013 Jul 08	*Miniopterus natalensis*	Genetic, Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3270	2013 Oct 02	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3271	2013 Oct 02	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3272	2013 Oct 02	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3273	2013 Oct 02	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3274	2013 Oct 02	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3435, TM50662	2013 Nov 05	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Bat had blood in mouth and nose.
UP 3465, TM50658	2013 Nov 05	*Cloeotis percivali*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Pregnant
UP 3563	2013 Nov 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3566	2013 Nov 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3567	2013 Nov 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3568	2013 Nov 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3569	2013 Nov 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3610	2013 Nov 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3664	2013 Nov 27	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3665	2013 Nov 27	*Hipposideros caffer*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3666	Not recorded	*Tadarida aegyptiaca*	Genetic	Gauteng	-25.751234, 28.276352	CSIR	Human bitten by bat

UP 3801; TM50567	2014 Jan 20	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3802; TM50564	2014 Jan 20	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead in cave
UP 3803	2014 Jan 21	*Miniopterus natalensis*	Morphological	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3804; NHCPHE MAM-36	2014 Jan 21	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat had a broken wing
UP 3805; TM50568	2014 Jan 21	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3815; NHCPHE MAM-37	2014 Jan 21	*Nycteris thebaica*	Genetic, Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3825; TM50569	2014 Jan 21	*Rhinolophus smithersi*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3826; NHCPHE MAM-38	2014 Jan 21	*Rhinolophus smithersi*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3830; TM50570	2014 Jan 21	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 3887, TM49146	2014 Feb 05	*Neoromicia (Laephotis) nana*	Genetic, Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3917, TM49148	2014 Feb 05	*Pipistrellus hesperidus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3918, TM48149	2014 Feb 05	*Pipistrellus hesperidus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 3920 , TM49168	2014 Feb 11	*Pipistrellus hesperidus*	Morphological	Mpumalanga	-25.71475, 28.98213	Blaauwpoort 257	None
UP 3921, TM49169	2014 Feb 12	*Neoromicia (Laephotis) capensis*	Museum voucher	Mpumalanga	-25.7113, 29.01755	Blaauwpoort 257	None
UP 3922, TM49170	2014 Feb 12	*Neoromicia (Laephotis) capensis*	Museum voucher	Mpumalanga	-25.72596, 28.99048	Blaauwpoort 257	None
UP 3923, TM49171	2014 Feb 12	*Neoromicia (Laephotis) capensis*	Museum voucher	Mpumalanga	-25.72596, 28.99048	Blaauwpoort 257	None
UP 3924, TM49172	2014 Feb 12	*Pipistrellus rusticus*	Morphological	Mpumalanga	-25.72596, 28.99048	Blaauwpoort 257	None
UP 3925, TM49173	2014 Feb 15	*Neoromicia (Laephotis) zuluensis*	Morphological	Mpumalanga	-25.65755, 29.01664	Blaauwpoort 257	None

UP 3926, TM49174	2014 Feb 21	*Rhinolophus clivosus*	Morphological	Mpumalanga	-25.6862, 29.0121	Blaauwpoort 257	None
UP 3927, TM49175	2014 Feb 22	*Myotis tricolor*	Morphological	Mpumalanga	-25.71454, 28.98284	Blaauwpoort 257	None
UP 3928	2009 Sep 30	*Neoromicia (Laephotis) capensis*	Genetic	Gauteng	-25.736022, 28.190374	Pretoria Zoo	None
UP 4105, NHCPHE MAM-73	2014 Apr 08	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4142, TM50565	2014 May 07	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4143, NHCPHE MAM-35	2014 May 07	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4150, TM50566	2014 May 07	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4165	2014 May 13	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4166	2014 May 13	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Bat had an infected ear
UP 4532; TM50590	2014 Sep 06	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4701, TM50636	2014 Nov 28	*Miniopterus natalensis*	Genetic, Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 4702, TM50637	2014 Nov 28	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.59751, 27.67145	Randstephne 455	None
UP 4749	2014 Dec 02	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4750	2014 Dec 02	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4830, TM49113	2015 Dec 01	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4831, TM49150	2015 Feb 01	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4840, TM49151	2015 Feb 01	*Hipposideros caffer*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4845, TM49152	2015 Feb 01	*Myotis tricolor*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None

UP 4903, TM49160	2015 Feb 02	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4904, TM49161	2015 Feb 02	*Myotis tricolor*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 4908, NHCPHE MAM-50	2015 Feb 23	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat had an injured wing and ear
UP 4909, TM50591	2015 Feb 23	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4918, NHCPHE MAM-51	2015 Feb 23	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4970, NHCPHE MAM-72	2015 Feb 27	*Myotis tricolor*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4971, NHCPHE MAM-52	2015 Feb 27	*Pipistrellus rusticus*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4972, TM49189	2015 Feb 28	*Neoromicia (Laephotis) capensis*	Museum voucher	Limpopo	-24.61737, 27.68773	Jonker trust farm	Bat found dead
UP 4974, TM50592	2015 Feb 27	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4985, NHCPHE MAM-54	2015 Mar 01	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4988, TM50593	2015 Mar 01	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 4997, NHCPHE MAM-53	2015 Mar 02	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5013; TM49069	2015 Mar 04	*Neoromicia (Laephotis) capensis*	Museum voucher	Limpopo	-24.625197, 27.697872	Jonker trust farm	None
UP 5014, TM49190	2015 Mar 04	*Neoromicia (Laephotis) capensis*	Museum voucher	Limpopo	-24.625197, 27.697872	Jonker trust farm	None
UP 5023, TM49164	2015 Apr 21	*Rhinolophus clivosus*	Genetic, Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 5024, TM49165	2015 Apr 21	*Rhinolophus clivosus*	Genetic, Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 5025, TM49166	2015 Apr 21	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None

UP 5026, TM49167	2015 Apr 21	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 5027, TM49176	2015 Apr 16	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5028, TM49177	2015 Apr 17	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5029, TM49178	2015 Apr 17	*Rhinolophus swinnyi*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5030, TM49179	2015 Apr 17	*Rhinolophus swinnyi*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5031, TM49180	2015 Apr 17	*Rhinolophus swinnyi*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5035, TM49181	2015 Apr 20	*Laephotis botswanae*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5036, TM49182	2015 Apr 20	*Neoromicia (Laephotis) capensis*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5038, TM49183	2015 Apr 20	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5039, TM49184	2015 Apr 21	*Rhinolophus clivosus*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	Necropsy identified an enlarged abdomen and testes
UP 5040, TM49195	2015 Apr 29	*Tadarida aegyptiaca*	Museum voucher	KwaZulu-Natal	-29.48694, 29.89806	Wakefield farm	None
UP 5060	2015 Mar 03	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5061	2015 Mar 03	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5066	2015 Mar 03	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5121	2015 Mar 04	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5134	2015 Mar 04	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5233	2015 May 06	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5275, TM49163	2015 May 06	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None

UP 5279, NHCPHE MAM-74	2015 May 11	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5287, NHCPHE MAM-75	2015 May 11	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5296, NHCPHE MAM-76	2015 May 11	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5304; TM49194	2015 May 11	*Neoromicia (Laephotis) capensis*	Museum voucher	KwaZulu-Natal	-29.331947, 30.27753	Welgevonden farm	None
UP 5319, NHCPHE MAM-77	2015 May 12	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5431, TM50664	2015 Jul 08	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Bat found dead
UP 5449, TM50663	2015 Jul 15	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	Bat found dead
UP 5450, TM50652	2015 Jul 15	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 5463, TM50594	2015 Jul 20	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Pregnant
UP 5467, NHCPHE MAM-55	2015 Jul 20	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead in tree
UP 5478, TM50595	2015 Jul 20	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5491, NHCPHE MAM-16	2015 Jul 21	*Nycteris thebaica*	Genetic, Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 5608	2015 Sep 02	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5619	2015 Sep 02	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5744, NHCPHE MAM-78	2015 Sep 16	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead in the grill of a car Necropsy identified internal organs rotten
UP 5784	2015 Sep 21	*Hipposideros caffer*	Morphological	Mpumalanga	-24.996531, 31.591762	Kruger National Park	None

UP 5830	2015 Oct 27	*Rhinolophus blasii*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Pregnant
UP 5842	2015 Oct 27	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Bat found dead
UP 5846	2015 Oct 27	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5927	2015 Oct 26	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Pregnant
UP 5928	2015 Oct 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5929	2015 Oct 26	*Miniopterus fraterculus*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5930	2015 Oct 26	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 5934	2015 Oct 27	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 6004, NHCPHE MAM-57	2015 Nov 10	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Pregnant, bat had a broken wing
UP 6076, TM50597	2015 Nov 12	*Cloeotis percivali*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Pregnant, bat had a broken wing
UP 6080, NHCPHE MAM-58	2015 Nov 12	*Cloeotis percivali*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6120	2016 Jan 12	*Myotis tricolor*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 6246, NHCPHE MAM-59	2016 Jan 19	*Miniopterus natalensis*	Genetic, Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6247, TM50598	2016 Jan 19	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6290, TM49236	2016 Jan 20	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6300, NHCPHE MAM-61	2016 Jan 20	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6302, TM50599	2016 Jan 20	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6381, TM50572	2016 Apr 05	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None

UP 6395, NHCPHE MAM-39	2016 Apr 07	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6399, TM50571	2016 Apr 07	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6795, TM50665	2016 Jun 06	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead in cave
UP 6814; NHCPHE MAM-17	2016 Jun 07	*Rhinolophus smithersi*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6826, NHCPHE MAM-42	2016 Jun 08	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 6848	2016 Jun 21	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7094, TM50574	2016 Sep 05	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead at the entrance of the cave
UP 7109; NHCPHE MAM-18	2016 Sep 06	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madivirology sammatle cave	None
UP 7161	2016 Sep 14	*Miniopterus natalensis*	Morphological	North West	-26.036079, 26.704976	Die Coetzee Boerdery	None
UP 7255, TM 50575	2016 Nov 07	*Miniopterus natalensis*	Morphological	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 7278; NHCPHE MAM-19	2016 Nov 08	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead in cave
UP 7301, TM50646	2016 Nov 14	*Miniopterus natalensis*	Genetic, Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7408	2017 Feb 16	*Miniopterus natalensis*	Morphological	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	Bat found dead in cave Necropsy identified blood in mouth, nose and hardened organs
UP7560	2017 Mar 28	*Rhinolophus clivosus*	Morphological	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 7561, TM50583	2017 Mar 28	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 7585, TM50584	2017 Mar 29	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 7766, TM50647	2017 May 03	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None

UP 7767, TM50648	2017 May 03	*Miniopterus natalensis*	Genetic, Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7770, TM50650	2017 May 03	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7771, TM50651	2017 May 03	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7880, NHCPHE MAM-69	2017 May 15	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7886, NHCPHE MAM-70	2017 May 15	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7905, NHCPHE MAM-71	2017 May 17	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 7922, NHCPHE MAM-62	2017 May 24	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 7933	2013 Jul 08	*Myotis tricolor*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7936	2013 Jul 08	*Myotis tricolor*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7937	2013 Sep 03	*Rhinolophus clivosus*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 7943, TM50644	2017 Aug 28	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat had a wing broken
UP 7949, TM50639	2017 Aug 28	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat found dead in cave
UP 7982, TM50655	2017 Sep 07	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	Bat found dead in cave
UP 7983, TM50656	2017 Sep 07	*Miniopterus natalensis*	Museum voucher	Gauteng	-25.897071, 28.221455	Grootboom cave, Irene	None
UP 8009	2017 Sep 26	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8054	2017 Sep 27	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8106	2017 Sep 27	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8107	2017 Sep 27	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Necropsy identified blood present in intestines

UP 8132	2017 Sep 28	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8162, TM50661	2017 Oct 16	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Pregnant
UP 8167, TM50660	2017 Oct 16	*Miniopterus fraterculus*	Genetic, Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Foetus of UP 8207
UP 8207, TM50654	2017 Oct 17	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8235	2017 Oct 17	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Pregnant
UP 8356	2017 Nov 07	*Miniopterus natalensis*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8368	2017 Nov 07	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8369	2017 Nov 07	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Newborn pup dropped in net
UP 8390	2017 Nov 07	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8437	2017 Nov 08	*Miniopterus natalensis*	Genetic	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Bat found dead in cave Necropsy identified black intestine and swolen abdomen
UP 8485, TM50577	2017 Nov 20	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 8488, TM50578	2017 Nov 20	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Foetus of UP 8555
UP 8502, TM50579	2017 Nov 21	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Foetus of UP 8540
UP 8540, TM50580	2017 Nov 23	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.61335, 27.65381	Randstephne 455	None
UP 8555, TM50581	2017 Nov 23	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.61335, 27.65381	Randstephne 455	Bat found dead in pool Necropsy identified swollen stomach
UP 8572, TM50657	2017 Dec 05	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8599	2016 Dec 01	*Pipistrellus rusticus*	Morphological	Gauteng	-25.8852, 28.3608	Grootfontein Country Estate	None
UP 8602	2018 Jan 24	*Myotis tricolor*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8614	2018 Jan 24	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None

UP 8615	2018 Jan 24	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8667	2018 Feb 07	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8668	2018 Feb 07	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8676	2018 Feb 07	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8679	2018 Feb 07	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8720, TM50604	2018 Feb 26	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 8747, NHCPHE MAM-63	2018 Feb 27	*Neoromicia (Laephotis) capensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Necropsy identified large spleen
UP 8748, TM50605	2018 Feb 27	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 8752, NHCPHE MAM-64	2018 Feb 27	*Nycteris thebaica*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 8816	2018 Mar 06	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8874	2018 Apr 10	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8876	2018 Apr 10	*Rhinolophus blasii*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8879	2018 Apr 10	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8929	2018 Apr 10	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 8932	2018 Apr 11	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 9029, TM50606	2018 May 21	*Rhinolophus blasii*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 9042, NHCPHE MAM-65	2018 May 21	*Rhinolophus smithersi*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 9060, TM50607	2018 May 22	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None

UP 9075, TM50608	2018 May 23	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Bat caught by dog with blood in mouth, nose and internal injuries. Bat gave birth to a pup after a few days of rehabilitation care. Bat and pup later died.
UP 9689	2018 Jun 06	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	Pup of UP 9075
UP 9745	2018 Jul 18	*Mops midas*	Morphological	Limpopo	-24.059627, 31.043614	Phalaborwa	Necropsy identified blood in mouth with a brown/white mottled stomach
UP 9746	2018 Jul 18	*Mops midas*	Morphological	Limpopo	-24.059627, 31.043614	Phalaborwa	None
UP 9777, TM50653	2018 Jul 31	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 9896, TM50640	2018 Aug 20	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Necropsy identified blood in mouth
UP 9938, TM50641	2018 Aug 21	*Miniopterus natalensis*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	Very old bat
UP 9946, TM50642	2018 Aug 22	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 9947, TM50643	2018 Aug 22	*Rhinolophus simulator*	Museum voucher	Limpopo	-24.6181, 27.65231	Madimatle cave	None
UP 9963	2018 Sep 04	*Rhinolophus clivosus*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 9964	2018 Sep 04	*Rhinolophus blasii*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 10018	2018 Sep 05	*Rhinolophus simulator*	Morphological	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 10029, TM50613	2018 Oct 02	*Rhinolophus clivosus*	Museum voucher	Limpopo	-24.11487, 30.12151	Matlapitsi cave	None
UP 11502, TM48415	2011 Mar 01	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.39905, 28.360783	Kwalata Game Ranch	None
UP 11503, TM48416	2011 Mar 02	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.39815, 28.35425	Kwalata Game Ranch	None
UP 11504, TM48432	2011 May 04	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.380267, 28.316867	Kwalata Game Ranch	None
UP 11505, TM48373	2011 Jan 06	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.389917, 28.322633	Kwalata Game Ranch	None
UP 11506, TM48387	2011 Jan 08	*Scotophilus dinganii*	Genetic, Museum voucher	Gauteng	.25.385033, 28.317483	Kwalata Game Ranch	None
UP 11507, TM48399	2011 Jan 15	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.38985, 28.316483	Kwalata Game Ranch	None

UP 11508, TM48422	2011 Apr 15	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.391733, 28.323133	Kwalata Game Ranch	None
UP 11509, TM48401	2011 Jan 16	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.387783, 28.322767	Kwalata Game Ranch	None
UP 11510, TM48433	2011 May 20	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.379083, 28.317467	Kwalata Game Ranch	None
UP 11511, TM48409	2011 Feb 23	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.41567, 28.312167	Kwalata Game Ranch	None
UP 11512, TM48421	2011 Mar 27	*Neoromicia (Laephotis) capensis*	Museum voucher	Gauteng	-25.389917, 28.322633	Kwalata Game Ranch	None
UP 11513, TM48408	2011 Jan 29	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.379833, 28.321717	Kwalata Game Ranch	None
UP 11514, TM48398	2011 Jan 14	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.4026, 28.337683	Kwalata Game Ranch	None
UP 11515, TM48391	2011 Jan 08	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.385033, 28.317483	Kwalata Game Ranch	None
UP 11516, TM48392	2011 Jan 08	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.385033, 28.317483	Kwalata Game Ranch	None
UP 11517, TM48393	2011 Jan 08	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.385033, 28.317483	Kwalata Game Ranch	None
UP 11518, TM48371	2011 Jan 04	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11519, TM48372	2011 Jan 04	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11520, TM48361	2010 Dec 15	*Laephotis botswanae*	Genetic, Museum voucher	Gauteng	-25.385133, 28.319967	Kwalata Game Ranch	None
UP 11521, TM48402	2011 Jan 16	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.387783, 28.322767	Kwalata Game Ranch	None

UP 11522, TM48362	2010 Dec 15	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.385133, 28.319967	Kwalata Game Ranch	None
UP 11523, TM48357	2010 Nov 30	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.398, 28.312017	Kwalata Game Ranch	None
UP 11524, TM48363	2010 Dec 15	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.385133, 28.319967	Kwalata Game Ranch	None
UP 11525, TM48364	2010 Dec 15	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.38533, 28.319967	Kwalata Game Ranch	None
UP 11526, TM48359	2010 Dec 02	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.381767, 28.3159	Kwalata Game Ranch	None
UP 11527, TM48365	2010 Dec 15	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.385133, 28.319967	Kwalata Game Ranch	None
UP 11528, TM48367	2011 Jan 04	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11529, TM48368	2011 Jan 04	*Scotophilus dinganii*	Genetic, Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11530, TM48369	2011 Jan 04	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11531, TM48403	2011 Jan 18	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.389917, 28.322633	Kwalata Game Ranch	None
UP 11532, TM48404	2011 Jan 18	*Pipistrellus hesperidus*	Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11533, TM48389	2011 Jan 08	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.385033, 28.317483	Kwalata Game Ranch	None
UP 11534, TM48390	2011 Jan 08	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.382567, 28.3172	Kwalata Game Ranch	None
UP 11535, TM48406	2011 Jan 22	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.39295, 28.3321	Kwalata Game Ranch	None

UP 11536, TM48396	2011 Jan 14	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.4026, 28.337683	Kwalata Game Ranch	None
UP 11537, TM48407	2011 Jan 22	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.39295, 28.3321	Kwalata Game Ranch	None
UP 11538, TM48397	2011 Jan 14	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.4026, 28.337683	Kwalata Game Ranch	None
UP 11539, TM48410	2011 Feb 23	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11540, TM48411	2011 Feb 23	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11541, TM48413	2011 Feb 23	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11542, TM48425	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11543, TM48426	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11544, TM48426	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11545, TM48426	2010 Dec 02	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11546, TM48360	2010 Dec 15	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11547, TM48366	2011 Jan 04	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11548, TM48370	2011 Jan 04	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 11549, TM48424	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None

UP 11550, TM48434	2011 May 20	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 12814, TM48435	2011 May 29	*Scotophilus dinganii*	Genetic, Museum voucher	Gauteng	-25.172, 28.366267	Kwalata Game Ranch	None
UP 12815, TM48381	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12816, TM48382	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12817, TM48417	2011 Mar 02	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.392950, 28.3321	Kwalata Game Ranch	None
UP 12818, TM48400	2011 Jan 15	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.389850, 28.316483	Kwalata Game Ranch	None
UP 12819, TM48385	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12820, TM48386	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12821, TM48418	2011 Mar 02	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.392950, 28.3321	Kwalata Game Ranch	None
UP 12822, TM48430	2011 Apr 19	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.382533, 28.3167	Kwalata Game Ranch	None
UP 12823, TM48412	2011 Feb 23	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 12824, TM48395	2011 Jan 14	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.402600, 28.337683	Kwalata Game Ranch	None
UP 12825, TM48405	2011 Jan 18	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.389917, 28.322633	Kwalata Game Ranch	None
UP 12826, TM48374	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None

UP 12827, TM48419	2011 Mar 13	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.402650, 28.3377	Kwalata Game Ranch	None
UP 12828, TM48436	2011 Jan 21	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.399933, 28.352517	Kwalata Game Ranch	None
UP 12829, TM48420	2011 Mar 26	*Pipistrellus rusticus*	Museum voucher	Gauteng	-25.399933, 28.352517	Kwalata Game Ranch	None
UP 12830, TM48380	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12831, TM48423	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.417083, 28.31165	Kwalata Game Ranch	None
UP 12832, TM48383	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12833, TM48384	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12834, TM48375	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12835, TM48394	2011 Jan 08	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.385033, 28.317483	Kwalata Game Ranch	None
UP 12836, TM48427	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.417083, 28.31165	Kwalata Game Ranch	None
UP 12837, TM48428	2011 Apr 19	*Tadarida aegyptiaca*	Genetic, Museum voucher	Gauteng	-25.417083, 28.31165	Kwalata Game Ranch	None
UP 12838, TM48431	2011 Apr 19	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.382533, 28.3167	Kwalata Game Ranch	None
UP 12839, TM48376	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12840, TM48377	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None

UP 12841, TM48378	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None
UP 12842, TM48414	2011 Feb 23	*Neoromicia (Laephotis) capensis*	Genetic, Museum voucher	Gauteng	-25.415667, 28.312167	Kwalata Game Ranch	None
UP 12843, TM48379	2011 Jan 07	*Nycteris thebaica*	Genetic, Museum voucher	Gauteng	-25.38095, 28.345017	Tamboti Lodge	None

Appendix B

Table B1 Ethical and regulatory documentation

Permits by Province	Permit no.	Expiry date	Issue date
Limpopo			
Waterberg game park	A02-005-00003	16/2/2005	16/2/2004
Lapalala and Makalali game reserve	CPM-005-00010	30/4/2007	30/3/2007
Limpopo-Pafuri- Kruger National Park- Skukuza	RB/2010/22	31/12/2010	5/10/2010
General Limpopo	001-CPM402-00007	8/2/2014	8/2/2013
Limpopo- Scientific research on mammals	ZA/LP/84188	29/08/2018	29/08/2017
General Limpopo	ZA/LP/83642	8/3/2018	8/3/2017
Limpopo- Scientific research on mammals	CPM006806	3/5/2017	3/5/2012
General Limpopo	ZA/LP/73972	5/2/2017	5/2/2016
Gauteng			
Gauteng- bats	CPF6 No.0027	28/4/2011	29/4/2010
Gauteng- bats	CPF6 No.0109	3/3/2016	04/03/2014
Import from Limpopo to Gauteng	CPB6 No.003767	24/11/2012	25/5/2012
Gauteng- bats	CPF6 No.0123	5/4/2018	6/4/2016
Import from Limpopo to Gauteng	CPB6 No.0800	18/10/2017	19/04/2017
North West			
Collection of bats	000039 NW-07	31/12/2007	19/2/2007
Waterberg bat collection	167	21/9/2001	14/9/2001
Mpumalanga			
Collect small mammals - Marloth park	MPB.5177	5/1/2007	05/01/2007
Telperion Mpumalanga portion	MPB.5385	31/12/2014	10/02/2014
KwaZulu Natal			
KZN wildlife permit	OP 2098/2015	17/05/2016	18/05/2015

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Disease ecology of pathogens in African bat species**
PROJECT NUMBER	**EC054-14 (revised)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Prof W Markotter**

STUDENT NUMBER (where applicable)	————————————————
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

Kindly note that a permit has to be submitted

ANIMAL SPECIES		**(a)Egyptian fruit bat**	**(b)Wahlberg's Epauletted fruit bat**
(c)Peter's Epauletted bat	**(d)Long fingered fruit bat**	**(e)Horseshoe bat**	**(f)Other**
NUMBER OF ANIMALS		**(a)150**	**(b)20**
(c)20	**(d)100**	**(e)20**	**(f)20**
Approval period to use animals for research/testing purposes			**2014 - 2018**
SUPERVISOR		**Prof. W Markotter**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 June 2014
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Disease ecology of pathogens in African bat species**
PROJECT NUMBER	**EC054-14 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Prof W Markotter**

STUDENT NUMBER (where applicable)	----------
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

Kindly note that a permit has to be submitted

ANIMAL SPECIES	**(a)Egyptian fruit bat; (b)Wahlberg's Epauletted fruit bat; (c)Peter's Epauletted fruit bat; (d)Long fingered fruit bat (e)Horseshoe bat; (f)Other**	
NUMBER OF ANIMALS	**(a; b; c; d; e) Submit a full report at the end of the study**	
Approval period to use animals for research/testing purposes		**October 2017-October 2018**
SUPERVISOR	**Prof. W Markotter**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 October 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Surveillance of the rabies-related Duvenhage virus in insectivorous bat species in South Africa**
PROJECT NUMBER	**H007-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**C Grobler**

STUDENT NUMBER (where applicable)	**U_14063329**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPESIES/SAMPLES	*1 Chaerephon ansorgei* *6 Chaerephon pumilus* *3 Cloeotis percivali* *2 Eptesicus hottentotus* *6 Glauconycteris variegatus* *29 Hipposideros caffer* *1 Kerivoula argentata* *1 Laephotis bostwanae* *751 Miniopterus natalensis* *7 Mops condylurus* *53 Myotis tricolor* *25 Neoromicia capensis* *3 Neoromicia helios* *8 Neoromicia nana* *1 Neoromicia rueppellii* *3 Neoromicia zuluensis* *266 Nycteris thebaica* *13 Nycticeinops schlieffeni* *8 Pipistrellus hesperidus* *3 Pipistrellus rusticus* *20 Rhinolophus blasii* *1 Rhinolophus capensis* *23 Rhinolophus clivosus* *1 Rhinolophus damarensis*	*2 Chaerephon ansorgei* *18 Chaerephon pumilus* *5 Cloeotis percivali* *1 Eptesicus hottentotus* *1 Eptesicus serotinus* *6 Glauconycteris variegatus* *19 Hipposideros caffer* *1 Kerivoula argentata* *1 Laephotis bostwanae* *111 Miniopterus natalensis* *8 Miniopterus schreibersii* *11 Mops condylurus* *1 Mops midas* *10 Myotis tricolor* *1 Myotis welwitschii* *28 Neoromicia capensis* *6 Neoromicia helios* *10 Neoromicia nana* *1 Neoromicia rueppellii* *3 Neoromicia zuluensis* *31 Nycteris thebaica* *13 Nycticeinops schlieffeni* *5 Otomops martiensseni* *2 Otomops spp.*

S4285-15

	5 Rhinolophus denti *1 Rhinolophus fumigatus* *3 Rhinolophus hildebrandtii* *1 Rhinolophus landeri* *145 Rhinolophus simulator* *12 Rhinolophus smithersi* *8 Rhinolophus spp.* *3 Rhinolophus swinnyi* *1 Sauromys petrophilus* *30 Scotophilus dinganii* *6 Scotophilus leucogaster* *2 Scotophilus spp.* *8 Scotophilus viridis* *5 Tadarida aegyptiaca* *2 Taphozous mauritianus*	*1 Pipidtrellus kuhli* *11 Pipistrellus hesperidus* *6 Pipistrellus nanus* *11 Pipistrellus rusticus* *1 Pipistrellus spp.* *7 Rhinolophus blasii* *1 Rhinolophus capensis* *17 Rhinolophus clivosus* *1 Rhinolophus damarensis* *9 Rhinolophus darlingi* *5 Rhinolophus denti* *2 Rhinolophus fumigatus* *3 Rhinolophus hildebrandtii* *1 Rhinolophus landeri* *36 Rhinolophus simulator* *2 Rhinolophus smithersi* *10 Rhinolophus spp.* *3 Rhinolophus swinnyi* *1 Sauromys petrophilus* *44 Scotophilus dinganii* *6 Scotophilus leucogaster* *1 Scotophilus nigrita* *5 Scotophilus spp.* *8 Scotophilus viridis* *2 Tadarida aegyptiaca* *4 Taphozous mauritianus*
NUMBER OF ANIMALS	**1467 Serum samples**	**493 Brain samples**
Approval period to use animals for research/testing purposes		**June 2018 - June 2019**
SUPERVISOR	**Prof. W Markotter**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	25 June 2018
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 November 2019

Approval Certificate
Amendment 2

AEC Reference No.:	H007-18
Title:	New title: Surveillance for rabies-related lyssaviruses in South African insectivorous bat species.
Researcher:	Mr CS Grobler
Student's Supervisor:	Prof W Markotter

Dear Mr CS Grobler,
The **Amendment** as supported by documents received between 2019-10-10 and 2019-11-05 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-11-05.

Please note the following about your ethics approval:

1. The title has been changed from Surveillance of the rabies-related lyssaviruses in South Africa bat species to New title: Surveillance for rabies-related lyssaviruses in South African insectivorous bat species.
2. Please remember to use your protocol number (H007-18) on any documents or correspondence with the AEC regarding your research.
3. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

16/08/2018

Approval Certificate
New Application

Ethics Reference No: 425/2018

Title: Surveillance of the rabies-related Duvenhage virus in insectivorous bat species in South Africa

Dear Mr Colyn Grobler

The **New Application** as supported by documents specified in your cover letter dated 12/07/2018 for your research received on the 12/07/2018, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 15/08/2018.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**425/2018**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60 / 61, 31 Bophelo Road, Gezina, Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

12 September 2019

Approval Certificate
Amendment

Ethics Reference No.: 425/2018

Title: Surveillance for rabies-related lyssaviruses in South African insectivorous bat species.

Dear Mr CS Grobler

The **Amendment** as supported by documents received between 2019-08-27 and 2019-09-11 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-09-11.

Please note the following about your ethics approval:

- Please remember to use your protocol number (425/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag X323
Arcadia 0007, South Africa
Tel +27 (0)12 356 3084
Email deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Professor Wanda Markotter

Centre of Viral Zoonosis

Faculty of Health Sciences

University of Pretoria

Email: wanda.markotter@up.ac.za

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Dear Professor Wanda Markotter

Your Email dated 3 March 2017, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
4. This Section 20 approval is only valid for the importation of samples as specified in point 5 below. For any samples to be collected locally, an application should be made for an

amendment to this Section 20 approval, to the Director Animal Health, and this application should be accompanied by a letter from the responsible state veterinarian; ,

5. A veterinary import permit must be obtained prior to importation of the following:
    a. Bat samples (faecal, urine, oral swabs, blood [serum], fur and wing biopsy's) from Mozambique, Zimbabwe, Namibia, Botswana, Swaziland, Lesotho, Angola, Zambia, Malawi, Madegascar and Reunion Island;
    b. Ectoparasites from bats from Mozambique, Zimbabwe, Namibia, Botswana, Swaziland, Lesotho, Angola, Zambia, Malawi, Madegascar and Reunion Island;
    c. Antibodies (mouse, bat and rat antibodies) from Bethyl Laboratories, USA;
    d. Virus reference strains (rabies and rabies related lyssaviruses) from centres for Disease control and Prevention (USA) and Animal and Plant Health Agency (UK);
    e. Synthetic constructs of genes (paramyxovirus, hantavirus, arenavirus, corona virus, lyssavirus, filovirus, leptospira, bartonella and rickettsia), from Genscript and GenArt (Canada)
    f. Cell lines (bat, domestic livestock and wildlife) from ATCC (USA)
    g. Beads coated with recombinant proteins from Department of Microbiology and Immunology, uniformed services, Bethesda (USA)
6. The areas from which the bat samples and ectoparasites from bats are collected as per points 5(a) and (b) above should not be under any disease restriction for diseases bats are susceptible to;
7. Imported bat samples, antibodies, virus reference strains, synthetic constructs of genes, cell lines, and beads coated with recombinant proteins must be flown to OR Tambo International Airport, South Africa. The responsible State Veterinarian must issue a Red Cross Permit for all samples to be transported directly to the National Institute of Communicable Diseases (NICD) BSL 4 laboratory;
8. Only Low risk and non-infectious samples destined for the BSL 3 (Centre for Viral Zoonosis) laboratory must leave the NICD BSL 4 laboratory for further research;
9. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the *National Road Traffic Act,* 1996 (*Act* No. 93 of 1996);
10. Study must be conducted in compliance with all the relevant permissions from the local nature conservation authorities;
11. Appropriate personal protective equipment must be worn when collecting samples or handling animals;
12. This Section 20 approval is valid for 3 years from the date of signature by the Director: Animal Health. An application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Disease ecology of zoonotic pathogens in bats

Researcher (s): Professor Wanda Markotter

Institution: Faculty of Health Sciences, University of Pretoria

Your Ref./ Project Number: ECO54-14

Our ref Number: 12/11/1/1/8

Kind regards,

Maja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2017 -05- 19

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Professor Wanda Markotter
Centre of Viral Zoonosis
University of Pretoria
Email: wanda.markotter@up.ac.za

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "DISEASE ECOLOGY OF ZOONOTIC PATHOGENS IN BATS"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Bat samples (faecal, urine, oral swabs, blood [serum], fur and wing biopsy's), and ectoparasites must be stored at the access controlled BSL 3, Centre for Viral Zoonosis at University of Pretoria in ultra-low temperature freezers;

ii) Stored bat samples may not be outsourced without prior written approval from DAFF;

iii) Should any bat samples be used for further research, written approval from the Director of Animal Health must be obtained prior to start of project;

iv) Extracted DNA/RNA samples may be distributed.

v) Extracted DNA/RNA samples may only be exported in full compliance with the requirements of the importing country.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date:

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Professor Wanda Markotter

Centre of Viral Zoonosis

Faculty of Health Sciences

University of Pretoria

Email: wanda.markotter@up.ac.za

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your email dated 22 May 2017, requesting an amendment to permission granted on 19 May 2017 under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following amended research/study, with the following conditions :

Conditions:

1. All conditions stipulated within the approval dated 2017-05-19 must still be complied with;
2. The only allowable deviation from the research protocol for which Section 20 approval was granted is the following:
3. Antibodies (mouse and rat antibodies), synthetic constructs of genes (paramyxovirus, hantavirus, arenavirus, corona virus, lyssavirus, filovirus, leptospira, bartonella and rickettsia), cell lines (Bovine, horse, sheep and swine) and beads coated with recombinant proteins may be transported directly to the BSL 3 laboratory of the Faculty of Health Sciences, University of Pretoria;

4. A veterinary import permit must be obtained prior to importation of bat tissue samples such as: lung, heart, brain, kidney, spleen, liver, rectum and intestines, from Mozambique, Zimbabwe, Namibia, Botswana, Swaziland, Lesotho, Angola, Zambia, Malawi, Madegascar and Reunion Island;
5. Sampling in South Africa may only occur in the area of KwaZulu-Natal province that has supplied a State Veterinary letter of no restriction to the researcher. No sampling may take place in any other area or province without written permission from the Director: Animal Health;

Title of research/study: Disease ecology of zoonotic pathogens in bats

Researcher (s): Professor Wanda Markotter

Institution: Faculty of Health Sciences, University of Pretoria

Your Ref./ Project Number: ECO54-14

Our ref Number: 12/11/1/1/8

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2017 -06- 0 6

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Name, Department of Agriculture, Forestry and Fisheries
Private Bag X250, Pretoria 0001

Enquiries: Mr Herry Gololo · Tel: 012 319 7532 · Fax: +27 12 319 7470 E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8

Professor Wanda Markotter
Centre of Viral Zoonosis
Faculty of Health Sciences
University of Pretoria

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your email dated 11 June 2019, requesting an amendment to the permission granted on 2017-05-19 under Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following amended research/study, with the following conditions:

Conditions:

1. All conditions stipulated in the approval dated 2017-05-19 (attached) must still be complied with;
2. The only allowable deviation is that influenza may be included as an additional pathogen subject to the compliance with the following:
   i) A veterinary import permit must be obtained prior to the importation of the six synthetic constructs of influenza A (H1N1, H3N2, H7N9, H9N2, H17N10, H18N11) in pUC57 plasmids from GenScript, USA;
   ii) The entire DNA/RNA extraction procedure must be conducted within the DAFF compliant BSL3 laboratory and only extracted DNA/RNA may be removed from the BSL3 laboratory.

Title of research/study: Disease ecology of zoonotic pathogens in bats
Researcher(s): Professor Wanda Markotter

Institution: Faculty of Health Sciences, University of Pretoria
Your Ref./ Project Number: ECO54-14
Our ref Number: 12/11/1/1/8

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2019 -07- 01

SUBJECT: **RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)**

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

CERTIFICATE OF COMPLIANCE

This is to certify that:

University of Pretoria (UP)

Centre for Viral Zoonoses (CVZ)

BSL3 Facility

DAFF Compliance Number:

DAFF-C05

is a DAFF Compliant BSL3 Facility (including rodent animal quarantine)

provided that all DAFF conditions and requirements are complied with.

The facility complies with the requirements of DAFF 001, DAFF 002 and DAFF 012.

Director: Animal Health

Initial DAFF Compliance Date: 25/04/2012

Certificate Commences: 07/10/2016

Certificate Expires: 07/10/2018

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

CERTIFICATE OF COMPLIANCE

This is to certify that:

University of Pretoria

Centre for Viral Zoonoses (CVZ)

BSL3 Facility

DAFF Compliance Number:

DAFF-C05

is a DAFF Compliant BSL3 Facility

provided that all DAFF conditions and requirements are complied with.

The facility complies with the requirements of Procedure Manual: Laboratory/Facility Biosafety and Biosecurity including Biobanks and Vector Protection and FORM: DAFF BSL 3

Director: Animal Health

Initial DAFF Compliance Date: 25/04/2012

Certificate Commences: 01/10/2018

Certificate Expires: 01/10/2020

G.P.S. 124-0907

PERMIT/LICENCE No.: 0000167

NORTHERN PROVINCE
DEPARTMENT OF AGRICULTURE, LAND AND ENVIRONMENT

PERMIT/LICENCE

[Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance No. 12 of 1983)]

DEON VON WIELLIGH
(Date stamp)
07 SEP 2001
REGULATORY SERVICES

Receipt No. N/A
Value: R N/A

Name and residential address of permit/licence holder:
Mr. E. SEAMARK
KENSINGTON, JOHANNESBURG - 2094

Name of land owner N/A

In terms of and subject to the provisions of the Nature Conservation Ordinance, 1983 (Ordinance No. 12 of 1983), and the regulations contained therein, the holder of this permit/licence is hereby authorised to TO COLLECT SMALL MAMALS FOR SCIENTIFIC PURPOSES

during the period of validity of this permit/licence, and subject to the conditions and requirements of this permit/licence.

SPECIES		SEX	NUMBER	TUSK/HORN No./ MICROCHIP No./ SAFRING No.	DESCRIPTION/DERIVATIVE
COMMON NAME	SCIENTIFIC NAME				
SAUROMYS / TAVERA / CHAEROPHON	EREMOPHORUS, TERRICOLUS, OTOMYS, HIPPOSIDEROS, MUSCARDINUS, MYOTIS, MUS, RHABDOMYS, LEPTOTIS, EPTESICUS, MICROPS		THREE SPECIMENS PER SPECIES		

	*ON	*FROM	*TO
PROPERTY			
NAME AND No.	WITTEBOOMEN		
MAGISTERIAL DISTRICT	WATERBERG		
PROVINCE	NORTHERN PROVINCE		
COUNTRY	RSA		

*Delete if not applicable.

Period of validity: From 14 / 9 / 001 To 21 / 9 / 001

For Member of Executive Council: Agriculture, Land and Environment

M.D. van Wielligh
Print name

Signature of Permit Holder

FIND ATTACHED TO THIS PERMIT/LICENCE THE NUMBER OF 6 SPECIAL CONDITIONS

PERMIT 000039 NW-07

Department of Agriculture, Conservation, Environment and Tourism

OFFICE OF THE SENIOR MANAGER: CONSERVATION SERVICES

ISSUED IN TERMS OF THE PROVISIONS OF THE TRANSVAAL NATURE CONSERVATION ORDINANCE 12/83, CAPE NATURE CONSERVATION ORDINANCE 19/74 AND THE BOPHUTHATSWANA NATURE CONSERVATION ACT 3 OF 1973.
In terms of and subject to the provisions of the Ordinances and Law and the regulations framed there under, the permit holder is hereby authorized subject to the conditions and requirements appearing on this permit to carry out the activity with the wild animals or plants as mentioned in the permit during the perioc of validity of this permit.

VALIDITY: FROM 19.02.2007 TO 31.12.2007

1	Description of permitted activity.	COLLECTION OF BATS FOR SCIENTIFIC PURPOSES
	Particulars of person: ID, Home address & telephone	Dr. Teresa Kearney Transvaal Museum, Vertebrate Department, 357 Botha Ave. Kloofsig, 0157 PO Box 413, Pretoria, 0001 Tel. 012 322 7632 Fax: 012 322 7939
	Venue of activity	North West Province
2	Species	Bats (Chiroptera)
5	Additional information	All results to be made available to NW DACE: Biodiversity Scientific Support – information may not be held back pending acceptance of publications. All information pertaining to specimens to be submitted in NW Biodiversity Information Management System format. Data sheets are available in electronic format from D Buijs. Progress reports to be submitted to D Buijs (dbuijs@nwpg.gov.za) and he must be informed about sampling trips (083 320 2727).

CONDITIONS AND REQUIREMENTS

THE PERMIT IS NOT TRANSFERABLE. ONLY A PERSON AUTHORIZED THERETO MAY MAKE AN ALTERATION ON THIS PERMIT. THIS PERMIT SHALL BE SUBJECT TO THE PROVISIONS OF ANY LAW IN FORCE DURING THE PERIOD OF VALIDITY OF THE PERMIT. THE HOLDER OF THE PERMIT WHO CONTRAVENES OR FAILS TO COMPLY WITH ANY ONE OF THE CONDITIONS OR REQUIREMENTS, TO WHICH THIS PERMIT IS SUBJECT, SHALL BE GUILTY OF AN OFFENCE. THE HOLDER OF THE PERMIT SHALL HAVE THE PERMIT WITH HIM/HER WHEN THE ACTIVITY IS CARRIED OUT

Drafted by
Permit Officer
Conservation Services
DACE: North West Province

Nature Conservation Permit Office
NORTH WEST PROVINCE
2007 -02- 19
DATE

Authorised by
Senior Manager / Manager Permitting
Conservation Services
DACE: North West Province

EZEMVELO KZN WILDLIFE

ORIGINAL

ORDINARY PERMIT

Fee: R 50.00
Receipt No: 2144/2015

Permit No: OP 2098/2015
Contact: Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 , Chapter 5 and the Regulations framed thereunder.

The permit is issued to:

ID Number: 6711250219082

Dr Teresa Kearney
Ditsong National of Natural History Vertebrate .
PO Box 413
432 Paul Kruger Street
Pretoria
0001

Residential Address
Ditsong National of Natural History Vertebrate .
PO Box 413
432 Paul Kruger Street
Pretoria
0001

Province: Gauteng

In the capacity of Researcher

To Capture and Export to another province the following species of Mammals

BATS
3 (Three) per species per locality Male

BATS
3 (Three) per species per locality Female

Collection permitted in Nottingham Road, Lions River District, throughout KwaZulu-Natal EXCLUDING KZN Wildlife protected areas

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 18 May 2015

for CHIEF EXECUTIVE

Kearney
Permit Holder

EZEMVELO KZN WILDLIFE PERMITS OFFICE
PO Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal.
Tel: +27 33 845 1370 / 1324, Fax: +27 33 845 1747, Fax to Email: 086 629 3320
Email: permits@kznwildlife.com, Website: www.kznwildlife.com

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 W 0269

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Mr. Ernest C J Seamark

Residential address: 351 Botha Avenue, Klopeig, 0181

DATE STAMP

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
To catch convey & Keep bat (Mammalia. Chiroptera) Specimens for Scientific purposes (as Three) Male & as Three) Females Voucher Specimen of each species See attached Conditions		

In terms of and subject of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983) and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

SIGNED ON BEHALF OF THE PREMIER

Valid until 19/07/2021
Valid until 19/07/2020

SIGNATURE OF PERMIT HOLDER

(See conditions on reverse side)

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 № 0123

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Mr Ernest C.J Seamark

Residential address: 357 Botha Avenue
Kloofsig
0157

GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT
06 APR 2016
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
P.O. BOX 8769, JOHANNESBURG 2000

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
To catch convey & keep bat specimens for Scientific Purposes.		
03 (Three) Male & 03 (Three) Female voucher specimen of each species		
See attached Conditions		

In terms of and subject of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983) and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

Ambhle Radebe

SIGNED ON BEHALF OF THE ~~PREMIER~~ MEC

SIGNATURE OF PERMIT HOLDER

Valid until 05-04-2018

(See conditions on reverse side)

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 № 0109

Addendum to catch, keep bats specimens

PERMIT TO HUNT AND/OR ~~COLLECT~~ AND CONVEY ~~A WILD ANIMALS~~
FOR SCIENTIFIC PURPOSES
Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Ernest Charles James

Residential address: Seamark
357 Botha Ave
Kloofsig
0157

DATE STAMP
GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT
2014 -03- 04
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PO BOX 8769 JOHANNESBURG 2000

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
To Catch, Convey and keep bat Specimens For Scientific Purposes.		
3(three) male and 3(three) female Voucher Specimens Of each species		
See attached Conditions		

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed
Co-workers working under supervision of Ernest			
Dr. Teresa C. Kearney (Ditsong National Museum)			
Dr Mark Keith (University of Pretoria)			
Prof. Wanda Markotter (University of Pretoria)			
Mr Steward D. McCulloch (University of Pretoria)			

Addendum / Valid: 03/03/2016

SIGNED ON BEHALF OF THE PREMIER MEC

SIGNATURE OF PERMIT HOLDER

(See conditions on reverse side)

PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 № 0027

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Ernest Charles J. Seamark

Residential address: 357 Botha Ave
Kloofsig
0157

DATE STAMP
GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE CONSERVATION, ENVIRONMENT
2010 -04- 29
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PO BOX 8769, JOHANNESBURG 2000

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
6(six)	Bats (Chiroptera)	3 Male
	SEE CONDITIONS ATTACHED	3 females

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animals referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

SIGNED ON BEHALF OF THE PREMIER

SIGNATURE OF PERMIT HOLDER

This Permit is valid from ~~28~~ 29/04/2010 to 28/04/2011

(See conditions on reverse side)

DEPARTMENT OF ECONOMIC DEVELOPMENT, ENVIRONMENT & TOURISM: LIMPOPO PROVINCE
DIRECTORATE: WILDLIFE TRADE AND REGULATION PERMIT

(Date Stamp) CITES & PERMIT MANAGEMENT ENVIRONMENTAL AFFAIRS LIMPOPO PROVINCE

[Issued in terms of the provisions of the Limpopo Environmental Management Act, 2003 (Act No. 7 of 2003)] **CPM 006806**

Receipt no. 1020399

Value R 50-00

Name of residential address of permit / licence holder: DR. WANDA MARKOTTER DEPT OF MICROBIOLOGY AND PLANT PATHOLOGY UNIVERSITY OF PRETORIA

Name of Land Owner:

In terms of and subject to the provisions of the Limpopo Environmental Management Act, 2003 (Act No. 7 of 2003) and the regulations contained therein, the holder of this permit / licence is hereby authorized to: DO SCIENTIFIC RESEARCH ON MAMMALS

during the period of validity of this permit, and subject to the conditions and requirements of this permit

SPECIES		SEX	NUMBER	TUSK/HORN No./ MICROCHIP No./ SAFRING No.	DESCRIPTION / DERIVATIVE
COMMON NAME	SCIENTIFIC NAME				
EGYPTIAN FRUIT BAT	ROUSETTUS AEGYPTIACUS				RESEARCH: DISEASE Ecology OF
Long Fingered insectivorous bat	MINIOPTERUS Species				ZOONOTIC VIRUSES ASSOCIATED WITH ROUSETTUS AEGYPTIACUS EGYPTIAN FRUIT BAT AND MINIOPTERUS SPECIES IN LIMPOPO, SOUTH AFRICA

*DELETE IF NOT APPLICABLE

		*FROM	*TO
PROPERTY	[JOHN GREAVES]		
NAME AND No.	LOUIS TRICHARDT AREA Soutpansberg, DYKE'S CAVE in MAGOEBASKLOOF, LEKGALAMEETSE N/R)		
DISTRICT	VHEMBE, CAPRICORN AND MOPANI		
LOCAL MUNICIPALITY			
PROVINCE	LIMPOPO		
COUNTRY	R.S.A		

Period of validity: From 03 : 05 : 2012 To 03 : 05 : 2017

for: Member of Executive Council
Economic Development, Environment and Tourism

Print name T. J. SEAKAMELA

Signature of Permit Holder

FIND ATTACHED TO THIS PERMIT THE NUMBER OF _____ SPECIAL CONDITIONS

AGREEMENT

BETWEEN

SOUTH AFRICAN NATIONAL PARKS
herein represented by **Dr. Freek Venter**
in his capacity as **Specialist Head of Department: Conservation Services**
(hereinafter referred to as "SANParks")

AND

MAKULEKE COMMUNAL PROPERTY ASSOCIATION
herein represented by Mr. Gezani Eric Tivani in his capacity as **Chairperson: Makuleke Communal Property Association**
(hereinafter referred to as 'Makuleke CPA")

AND

Dr. TC Kearney
6711250219082
(hereinafter referred to as "the Researcher")

WHEREAS the Researcher submitted a research proposal to Makuleke CPA to conduct a research on "**Inventory of bat species occurring at Pafuri (Makuleke Contractual Park), with a comparison of morphological and**

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF
ECONOMIC DEVELOPMENT, ENVIRONMENT & TOURISM

DO SCIENTIFIC RESEARCH ON MAMMALS

(Issued in terms of the provisions of the Limpopo Environmental Management Act 2003, Act no.7 of 2003).
In terms of and subject to the provisions of the abovementioned legislation and the regulation framed thereunder, the holder of this permit is hereby authorized to catch and/or collect the species and number of mammals specified on the table below for scientific purpose in the property mentioned on this permit.

Permit Holder

Name	PROF. WANDA MARKOTTER	
Trade Name	N/A	
ID/Passport Number	7406270122080	
Address [Physical | Postal]	ROOM 2-72, PATHOLOGY BUILDING, 5 BOPHELO ROAD, PRINSHOF CAMPUS,UNIVERSITY OF PRETORIA, CORNER OF STEVE BIKO AND DR SAVAGE ST, PRETORIA 0001	ROOM 2-72, PATHOLOGY BUILDING, PRINSHOF CAMPUS,UNIVERSITY OF PRETORIA, UNIVERSITY OF PRETORIA, P.O.BOX 323, ARCADIA,0007, RSA

Permit Details

Permit No :	ZA/LP/100499		
Reference No :	CPM/29253/2019		
Date Issued :	2019-10-04	Stamp:	CITES & PERMIT MANAGEMENT ENVIRONMENTAL AFFAIRS LIMPOPO PROVINCE
Valid until :	2020-10-04		
Paid (ZAR):	R 55.00		
Receipt No :	1130327		

Farm Name / Organization	District	Province	Country
	N/A	Limpopo	South Africa

See Special Condition

Species Name	Scientific Name	Quantity	Note

Printed by:Seakamela TJ	2019-10-04	2019-10-04	
	Printed Date:	**Effective Date:**	**Signature of Permit Holder**

I acknowledge, accept and understand fully the permit conditions as described.

GENERAL CONDITIONS AND REQUIREMENTS OF PERMIT/LICENSE/CERTIFICATE

1. This permit or certificate shall not be transferable.
2. Any unauthorized alterations to this permit, or certificate shall invalidate it.
3. This permit or certificate shall be subject to the provisions of any law in force during the period of validity of the permit or certificate, in the area to which the permit or certificate to such person.
4. The holder of this permit or certificate shall, at the request of a person authorized in terms of the relevant legislation so to demand, forthwith produce such permit, or certificate to such person.
5. The holder of this permit or certificate shall return this original, permit or certificate to the Director: Wildlife Trade and Regulation, Limpopo Province, P.O. Box 55464, Polokwane, 0700.
6. This permit or certificate shall be invalid until the signature of the holder thereof has been appended thereto.
7. This permit or certificate shall lapse when it is lost or destroyed and no copy thereof shall be issued.
8. The holder of this permit, or certificate who contravenes or fails to comply with any one of the contravenes or fails to comply with any one of the conditions or requirements to which this permit or certificate is subject, shall be guilty of an offence.
9. An officer authorized thereto by the MEC may cancel this permit or certificate at any time.
10. This permit or certificate does not absolve the holder thereof from the necessity of obtaining such other permits and/or documents as may be required by law from the relevant, Dept., Provincial or Country.
11. This permit, or certificate stays the property of the Department.
12. An officer authorized thereto by the MEC may cancel, alter or change any general conditions and requirements of the permit or certificate or any special condition attached to the permit or certificate.
13. The holder of this permit or certificate will collect the original permit, or certificate from LEDET's offices where the application was lodged.
14. The holder of this permit or certificate will sign the permit, or certificate and a copy of the permit, or certificate will either be couriered, e-mailed, faxed or hand delivered back to the office where the permit, or certificate was issued from, within five (5) working days upon receipt of the original permit, or certificate.

SPECIAL CONDITIONS

1. THIS PERMIT MUST BE SIGNED BY THE PERMIT HOLDER AND MUST BE IN HIS/HER POSSESSION FOR THE DURATION OF THE PROJECT.
2. THE PROJECT LEADER MUST OBTAIN THE WRITTEN PERMISSION OF THE LANDOWNER ON WHOSE PROPERTY THE ANIMALS WILL BE COLLECTED PRIOR TO THE COLLECTION THEREOF.
3. THIS PERMIT ALSO AUTHORISES THE HOLDER THEREOF TO CONVEY WITHIN THE PROVINCE, OR EXPORT FROM THE PROVINCE THE COLLECTED SPECIES REFER TO ON THIS PERMIT, TO ANY OTHER PROVINCE WITHIN THE COUNTRY. SUCH ACTION WILL BE SUBJECT TO IMPORT PERMITS FROM SUCH PROVINCE.
4. COPIES OF PUBLICATIONS EMANATING FROM THIS RESEARCH PROJECT SHOULD ALSO BE FORWARDED TO THE ADDRESS STATED UNDER POINT 6.
5. A DATASHEET ACCORDING TO THE ATTACHED DATA FORMAT MUST BE COMPLETED FOR EACH SPECIMEN COLLECTED AND BE RETURNED TO THE BIODIVERSITY OFFICE FOR THE PROVINCIAL BIOBASE PROJECT.
6. ALL REQUESTED INFORMATION OR ENQUIRIES MUST BE DIRECTED TO THE LIMPOPO ENVIRONMENTAL MANAGEMENT AUTHORITY, P.O. BOX 55464, POLOKWANE, 0700 .
7. THIS PERMIT IS ISSUED FOR MENTIONED SPECIES EXCLUDING ANY ENDANGERED SPECIES. (EGYPTIAN FRUIT BAT)

CITES & PERMIT MANAGEMENT
ENVIRONMENTAL AFFAIRS
LIMPOPO PROVINCE

Mpumalanga
TOURISM AND PARKS AGENCY

MPUMALANGA TOURISM AND PARKS AGENCY
RECEIVED
PRIVATE X 11338 NELSPRUIT 1200
TEL 013 759 5300/1
2014 -02- 10
FAX 013 759 5490
WILDLIFE PROTECTION SERVICES
PERMITS

MPB. 5385

PERMIT

TO ~~HUNT~~/CATCH / COLLECT AND CONVEY ANIMALS FOR SCIENTIFIC PURPOSE
(Issued in terms of the provisions of the Nature Conservation Act 10 of 1998)

Name and residential address of permit holder: *ECJ Seamark*
357 Botha Street
Kloofsig 0157

Dr. T. C. Kearney; Dr. M. Keith
Prof. W. Markotter; Mr. S. D McCulloch
Mr. L. Lebuschagne

Name and address of institution or department of whose behalf shall be hunted and conveyed: *AfricanBats*
KLOOFSIG

PARTICULARS OF ANIMALS WHICH MAY BE ~~HUNTED~~/CATCH / COLLECTED

Number	Species
	Bats species:– (*Chiroptera*)

PLACE: Telperion/Ezemvelo NR **(MPUMALANGA portion)**

In terms of and subject to the provisions of the Nature Conservation Act (Act No. 10 Of 1998) and the regulations framed thereunder , the above-mentioned person is hereby authorised, subject to the conditions and requirements appearing and this permit, to ~~hunt~~/catch/collected the animals referred to above during the period of validity of this permit on behalf of the institution or department referred to above

- **Bats individuals captured and not collected for further scientific analyses, be released as soon as possible after recording/sampling have been done, at the site of capture**
- **A list of all species that are captured and positively identified(with the relevant point locality data(GPS points), & copies of scientific report or articles be forwarded to MPTA, Terrestrial Ecosystems, Lydenburg**
- **Specimens collected for identification & research purposes, be deposited at the Ditsong National Museum of Natural History(to obtain a collection number)**
- **The following number of voucher specimens per known species will be allowed(consideration be given to the population size and conservation status of such species:**
  a) **Known species: Single male and female**
  b) **New species or species New to S.A.: Single male and female i**
  c) **Potentially cryptic species: Single male and female**
- **No pregnant or lactating females to be collected**
- **University research projects undertaken are subject to ethical clearance from relevant institution's ethical committee**
- **If caves are found, sensitivity of interior (formations and fauna) first be determined. Decision to proceed with research should be based on sensitivity and potential impact of disturbance on cave interior**
- **Should research proceed, cave protocols then be strictly adhered to.**
- **No monitoring equipment to be mounted on cave formations**
- **Permit holder must obtain written permission from legal owner to conduct research**

Period of validity of permit: From date of issue to: *31 December 2014*

.....
for CHIEF EXECUTIVE OFFICER

.....
Signature of permit holder

WILDLIFE PROTECTION SERVICES DIVISION
Private Bag x 11338 Nelspruit, 1200
Tel: +27 (13) 759 5300/1 Fax: +27 (13) 759 5490

SEE CONDITIONS AND REQUIREMENTS ON REVERSE SIDE

MPUMALANGA TOURISM AUTHORITY
Province of Mpumalanga

Mpumalanga
Tourism and Parks Agency

MPUMALANGA parks board
Republic of South Africa

MPB. 5177

PERMIT

TO ~~HUNT~~/CATCH / COLLECT AND CONVEY MAMMALS FOR SCIENTIFIC PURPOSE
(Issued in terms of the provisions of the Nature Conservation Act 10 of 1998)

2006 -12- 14

Name and residential address of permit holder: ***Dr. T. Kearney; Dr. S. Goodman; Mr. E. Seamark***
Transvaal Museum
P.O. Box 413
Pretoria 0002

Name and address of institution or department of whose behalf shall be hunted and conveyed: ***Transvaal Museum***
PRETORIA

PARTICULARS OF MAMMALS WHICH MAY BE ~~HUNTED~~/CATCH / COLLECTED

Number	Species
	See attached list of species that are allowed

PLACE: Marloth park Area

In terms of and subject to the provisions of the Nature Conservation Act (Act No. 10 Of 1998) and the regulations framed thereunder , the above-mentioned person is hereby authorised, subject to the conditions and requirements appearing and this permit, to ~~hunt~~/catch/collected and convey insects animals referred to above during the period of validity of this permit on behalf of the institution or department referred to above

- **A list of all species that are captured and positively identified (with the relevant point locality data (GPS points), be forwarded to Lientjie Cohen, Scientific Section, Terrestrial Ecosystems, Lydenburg.**
- **Copies of scientific reports or articles be send to the same office as mentioned above.**
- **Wing tissue (with a biopsy punch) can be taken from all the captured individuals.**
- **Should species which are cryptic and difficult to identify in the field (including potentially new species) be captured, the Mpumalanga Tourism and Parks Agency requests the permit holders to use discretion in terms of the numbers of individuals collected.**

Period of validity of permit: From date of issue to: ***05 January 2007***

.....
for CHIEF EXECUTIVE OFFICER

.....
Signature of permit holder

WILDLIFE PROTECTION SERVICES DIVISION
PRIVATE BAG X11338 • NELSPRUIT 1200
TEL +27 (0) 13 759 5300/1 • FAX +27 (0) 13 759 5498 • E-MAIL: Dorothy.Mnisi@pbroard1.agric.za

SEE CONDITIONS AND REQUIREMENTS ON REVERSE SIDE

Appendix C

Tabel C1 Details of sequences used for phylogenetic analysis

Genbank accession number	Virus	Host	Year	Country
KT336437	Rabies virus	*Canis lupus*	2012	South Africa
MG458308	Rabies virus	*Atilax paludinosus*	2000	South Africa
FJ392391	Rabies virus	*Mus musculus*	1994	South Africa
GQ918139	Rabies virus	CVS lab strain	2010	
LC325820	Rabies virus	CVS lab strain	2010	
NC025377	West Caucasian bat virus	*Miniopterus schreibersi*	2002	Russia
JX193798	Ikoma lyssavirus	*Civettictis civetta*	2009	Kenya
NC03195	Lleida bat lyssavirus	*Miniopterus schreibersii*	2011	Spain
HM179509	Lagos bat virus	*Epomophorus wahlbergi*	2008	South Africa
LN849915	Lagos bat virus	*Eidolon helvum*	2011	Ghana
EF547459	Lagos bat virus	*Eidolon helvum*	1956	Nigeria
MH643893	Lagos bat virus	*Epomophorus wahlbergi*	2016	South Africa
JX901139	Lagos bat virus	*Rousettus aegyptiacus*	2010	Kenya
DQ499948	Lagos bat virus	*Atilax paludinosus*	2004	South Africa
KF155008	Mokola virus	*Felis catus*	1996	South Africa
EU293118	Mokola virus	*Rodent*	1981	Central African Republic
KF155006	Mokola virus	*Felis catus*	1982	Zimbabwe
KF155005	Mokola virus	*Crocidura sp*	1968	Nigeria
NC025365	Shimoni bat virus	*Macronycteris vittatus*	2009	Kenya
AY573964	Australian bat lyssavirus	*Pteropus alecto*	1996	Australia
AF418014	Australian bat lyssavirus	*Homo sapiens*	1999	Australia
NC020808	Aravan virus	*Myotis blythi*	1991	Kyrgyzstan
JF311903	Bokeloh bat lyssavirus	*Myotis nattererii*	2010	Germany
MF043188	Bokeloh bat lyssavirus	*Myotis nattereri*	2013	France
EU293119	Duvenhage virus	*Homo sapiens*	1971	South africa
EU293120	Duvenhage virus	*Miniopterus* sp	1981	South africa
EU623437	Duvenhage virus	*Homo sapiens*	1970	South africa
EU623444	Duvenhage virus	Not identified	2006	South africa
AY863364	European bat lyssavirus 1	*Eptesicus serotinus*	2000	Netherlands
KP241939	European bat lyssavirus 1	*Eptesicus isabellinus*	2007	Spain
NC009528	European bat lyssavirus 2	*Homo sapiens*	2002	United Kingdom
NC031988	Gannoruwa bat lyssavirus	*Pteropus giganteus*	2015	Sri lanka
NC020809	Irkut virus	*Murina leucogaster*	2002	Siberia
MF960865	Kotalahti bat lyssavirus	*Myotis* sp	2017	Finland
NC025385	Khujand virus	*Myotis mystacinus*	2001	Tajikistan
MF472709	Taiwan bat lyssavirus	*Pipistrellus abramus*	2017	Taiwan
MF472710	Taiwan bat lyssavirus	*Pipistrellus abramus*	2016	Taiwan

Appendix D

Bayesian phylogeny and maximum likelihood analyses trees for lyssavirus positive sample UP 5619 for the P-, M-, G-, and L-genes

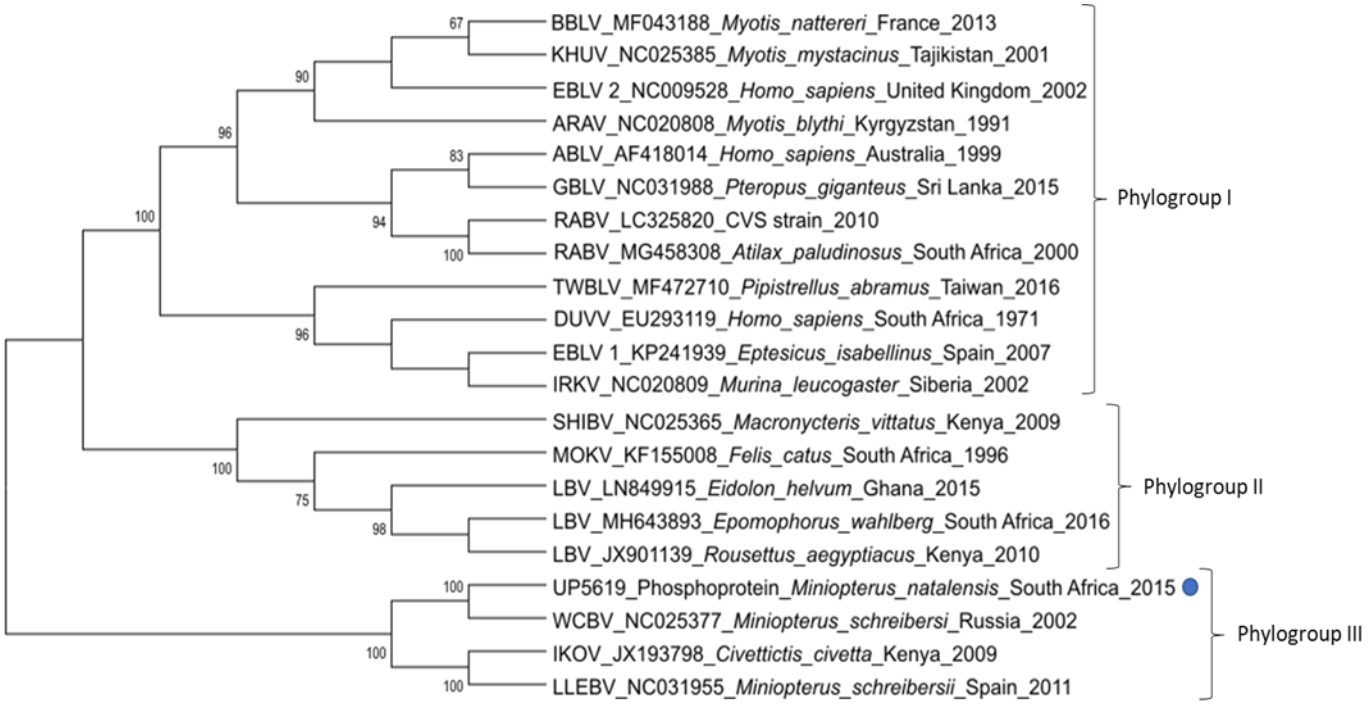


Figure D1 Maximum likelihood analysis of the relatedness of the lyssavirus full P gene of the one positive sample generated in this study, constructed using MEGA X using the GTR+G+I model. The analysis is based on all concatenated coding regions of the lyssavirus gene sequences with a bootstrap of 1000 replicates. Only bootstrap values above 50 are shown. Lyssavirus positive samples detected in this study were marked with blue dots.

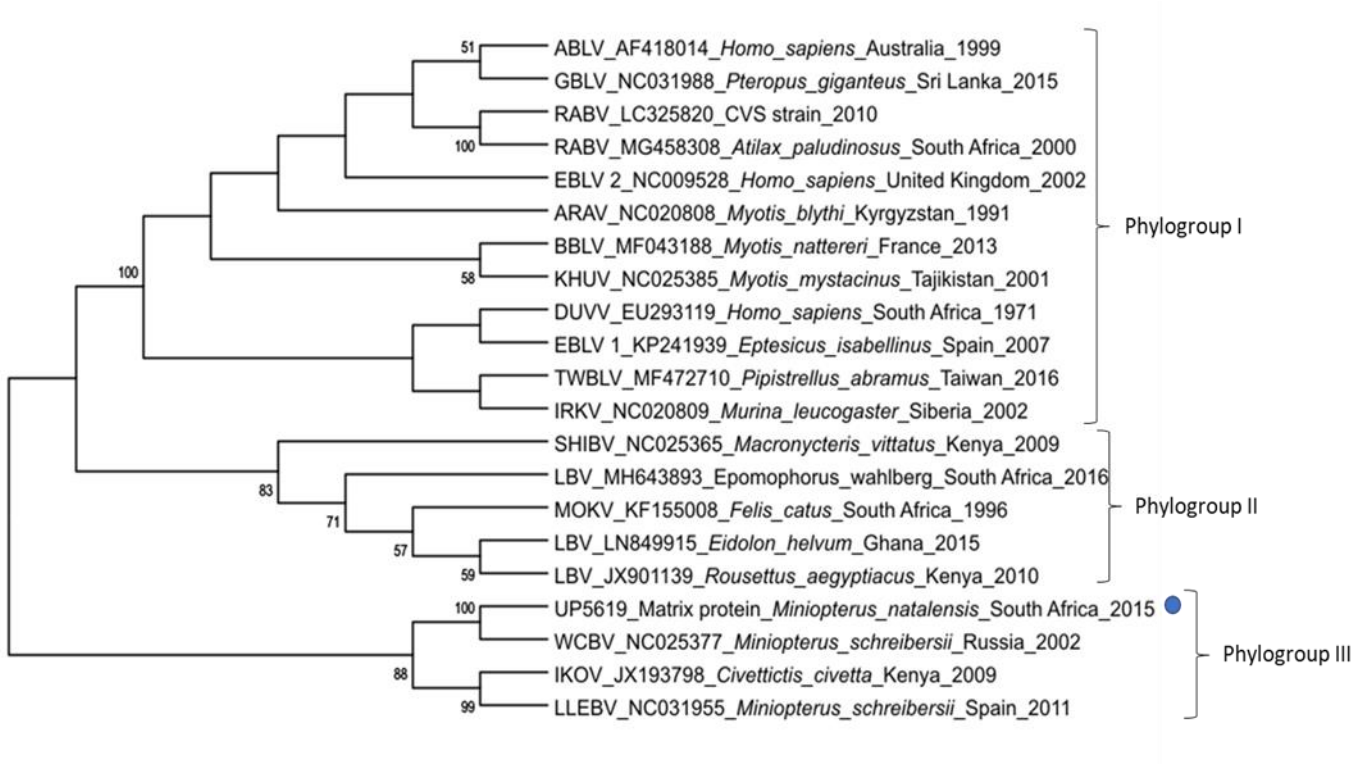


Figure D2 Maximum likelihood analysis of the relatedness of the lyssavirus full M gene of the one positive sample generated in this study, constructed using MEGA X using the GTR+G+I model. The analysis is based on all concatenated coding regions of the lyssavirus gene sequences with a bootstrap of 1000 replicates. Only bootstrap values above 50 are shown. Lyssavirus positive samples detected in this study were marked with blue dots.

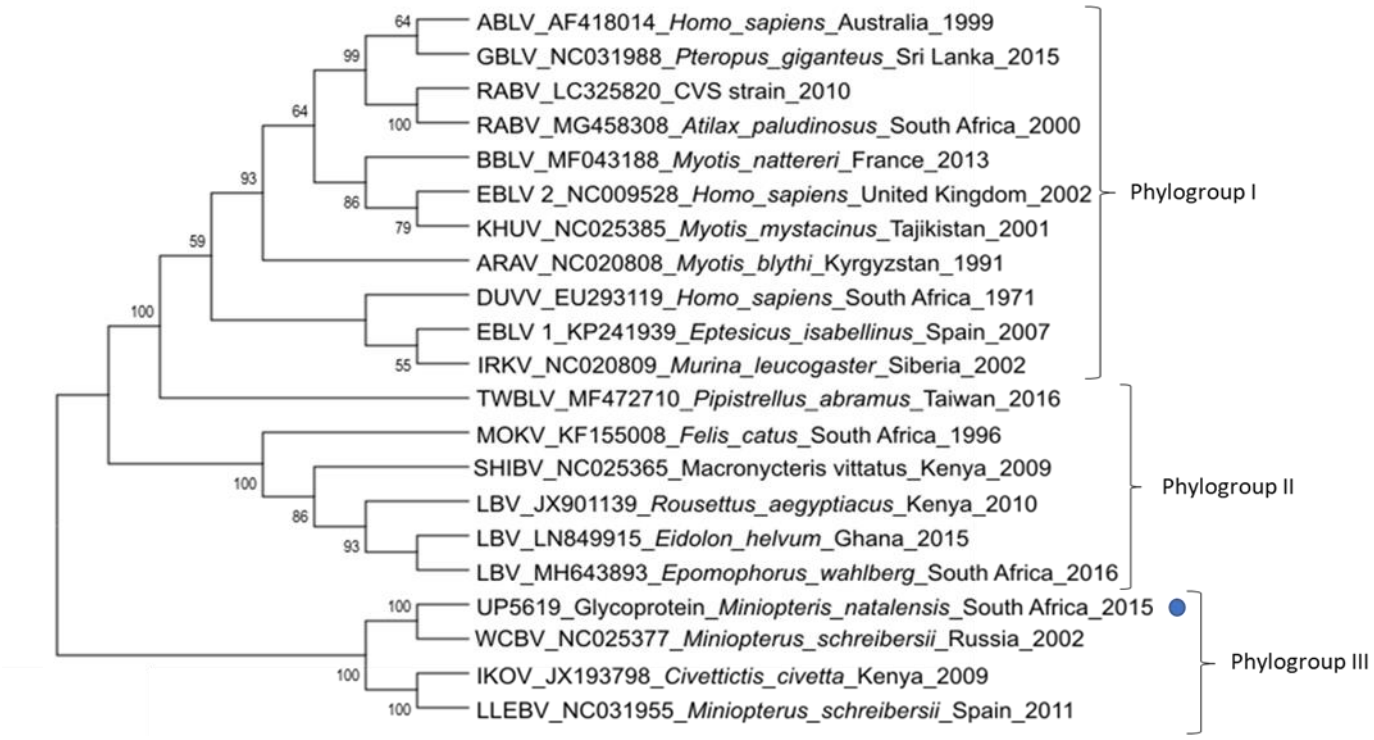


Figure D3 Maximum likelihood analysis of the relatedness of the lyssavirus full G gene of the one positive sample generated in this study, constructed using MEGA X using the GTR+G+I model. The analysis is based on all concatenated coding regions of the lyssavirus gene sequences with a bootstrap of 1000 replicates. Only bootstrap values above 50 are shown. Lyssavirus positive samples detected in this study were marked with blue dots.

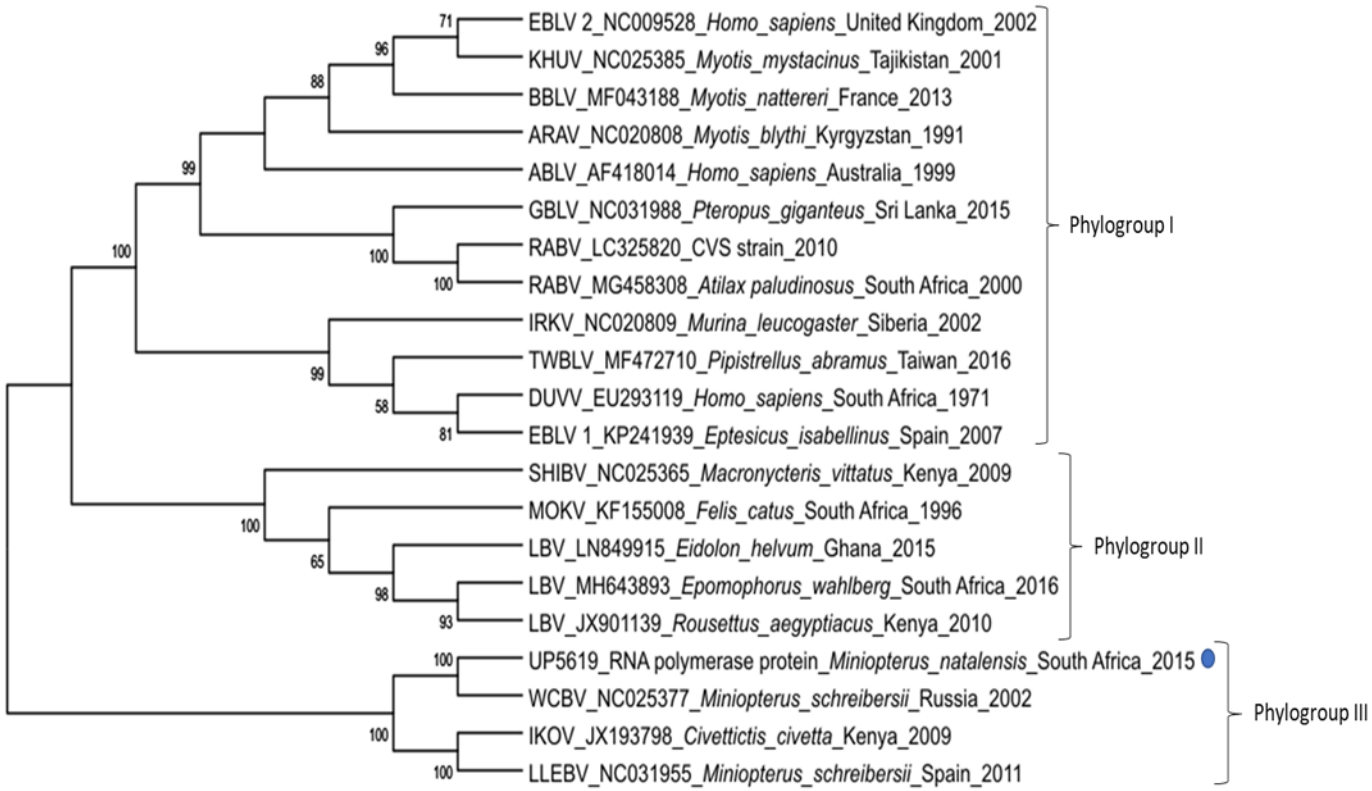


Figure D4 Maximum likelihood analysis of the relatedness of the lyssavirus full L gene of the one positive sample generated in this study, constructed using MEGA X using the GTR+G+I model. The analysis is based on all concatenated coding regions of the lyssavirus gene sequences with a bootstrap of 1000 replicates. Only bootstrap values above 50 are shown. Lyssavirus positive samples detected in this study were marked with blue dots

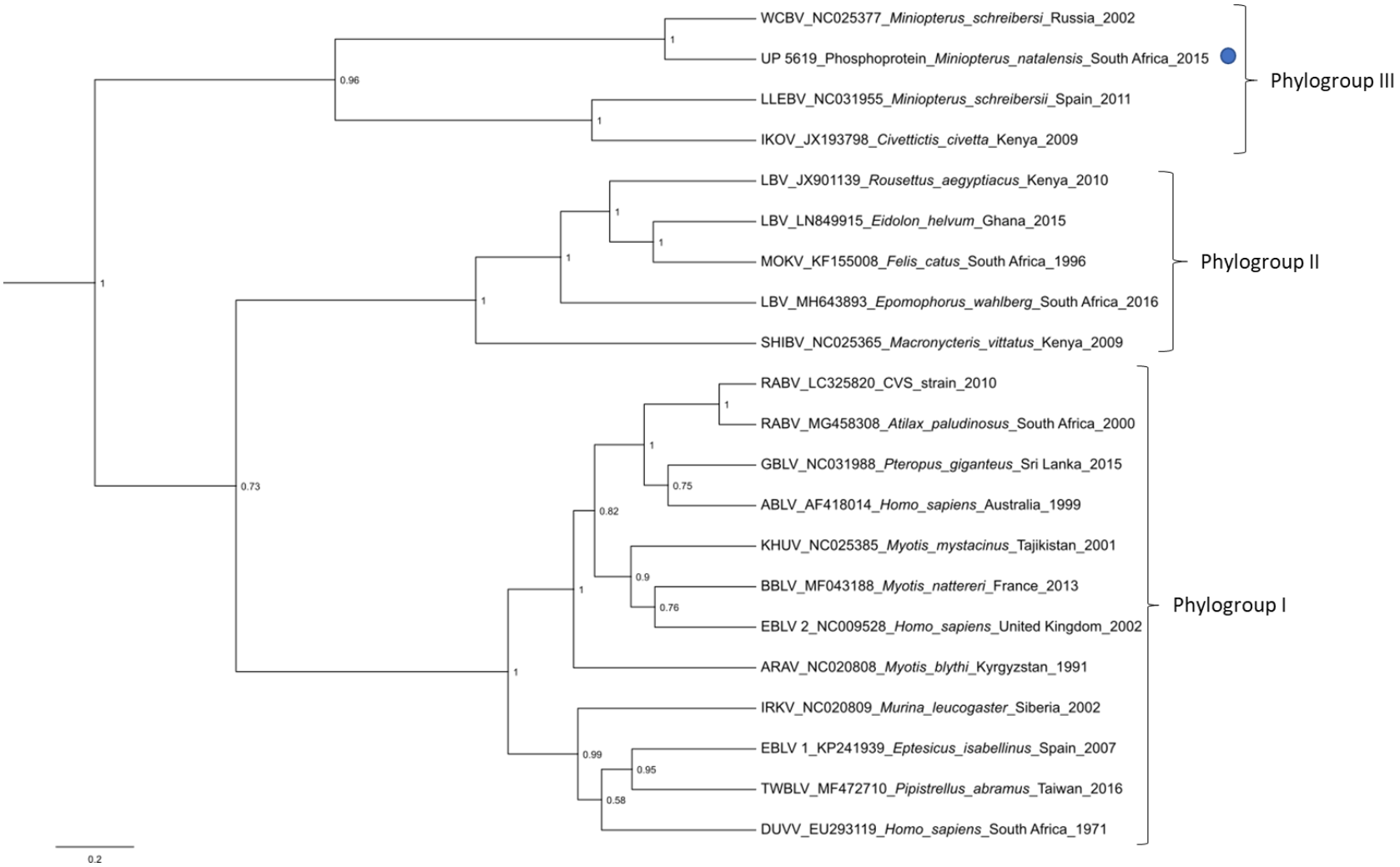


Figure D5 Phylogenetic analyses of the lyssavirus positive sample full P gene sequences generated in this study. Bayesian phylogeny of all concatenated coding regions of the lyssavirus gene sequences was achieved using BEAST employing the GTR+I+G substitution model for posterior probabilities at MCMC chain setting of 10M samples every 1000 states. Lyssavirus positive sample detected in this study are marked with a blue dot.

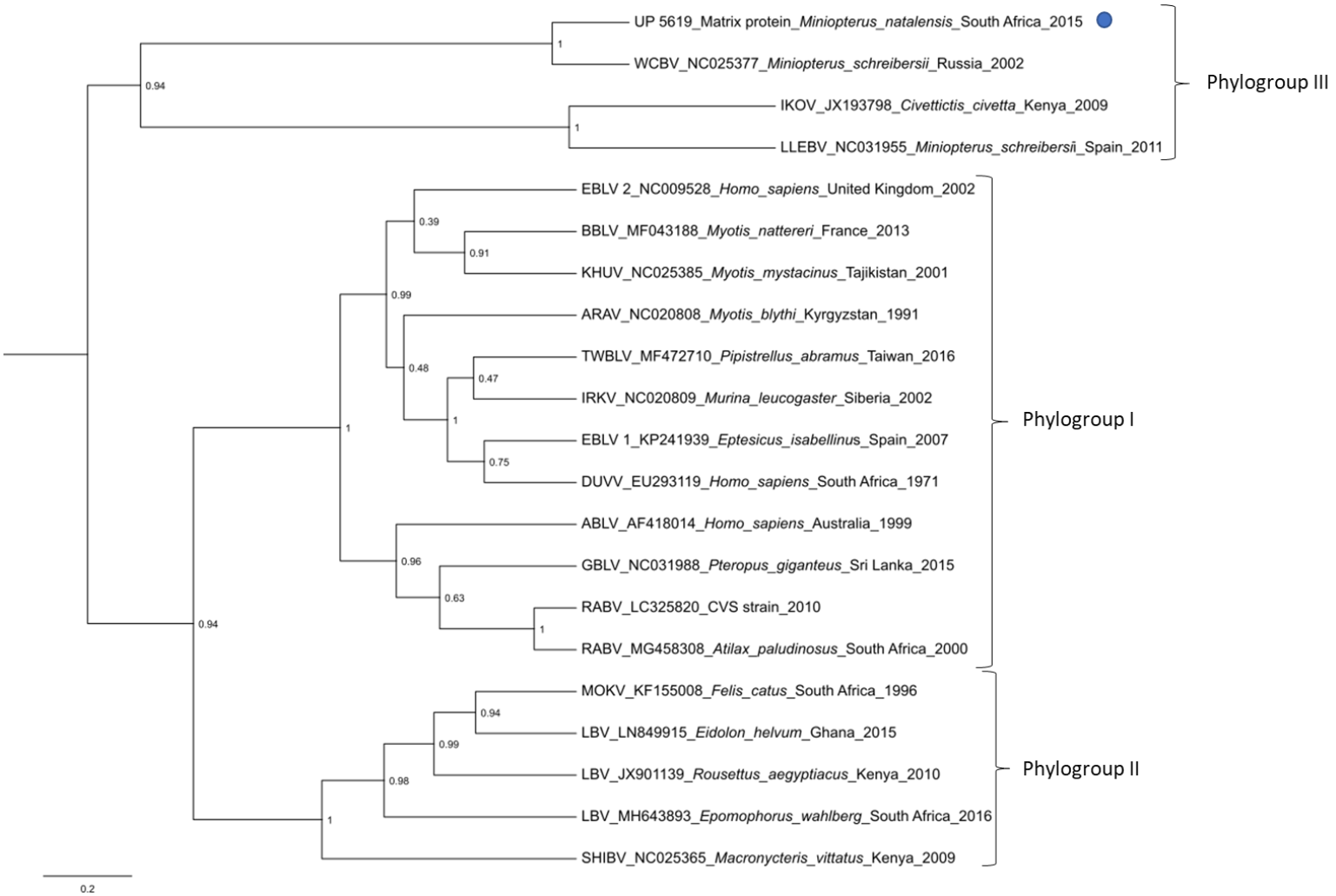


Figure D6 Phylogenetic analyses of the lyssavirus positive sample full M gene sequences generated in this study. Bayesian phylogeny of all concatenated coding regions of the lyssavirus gene sequences was achieved using BEAST employing the GTR+I+G substitution model for posterior probabilities at MCMC chain setting of 10M samples every 1000 states. Lyssavirus positive sample detected in this study are marked with a blue dot.

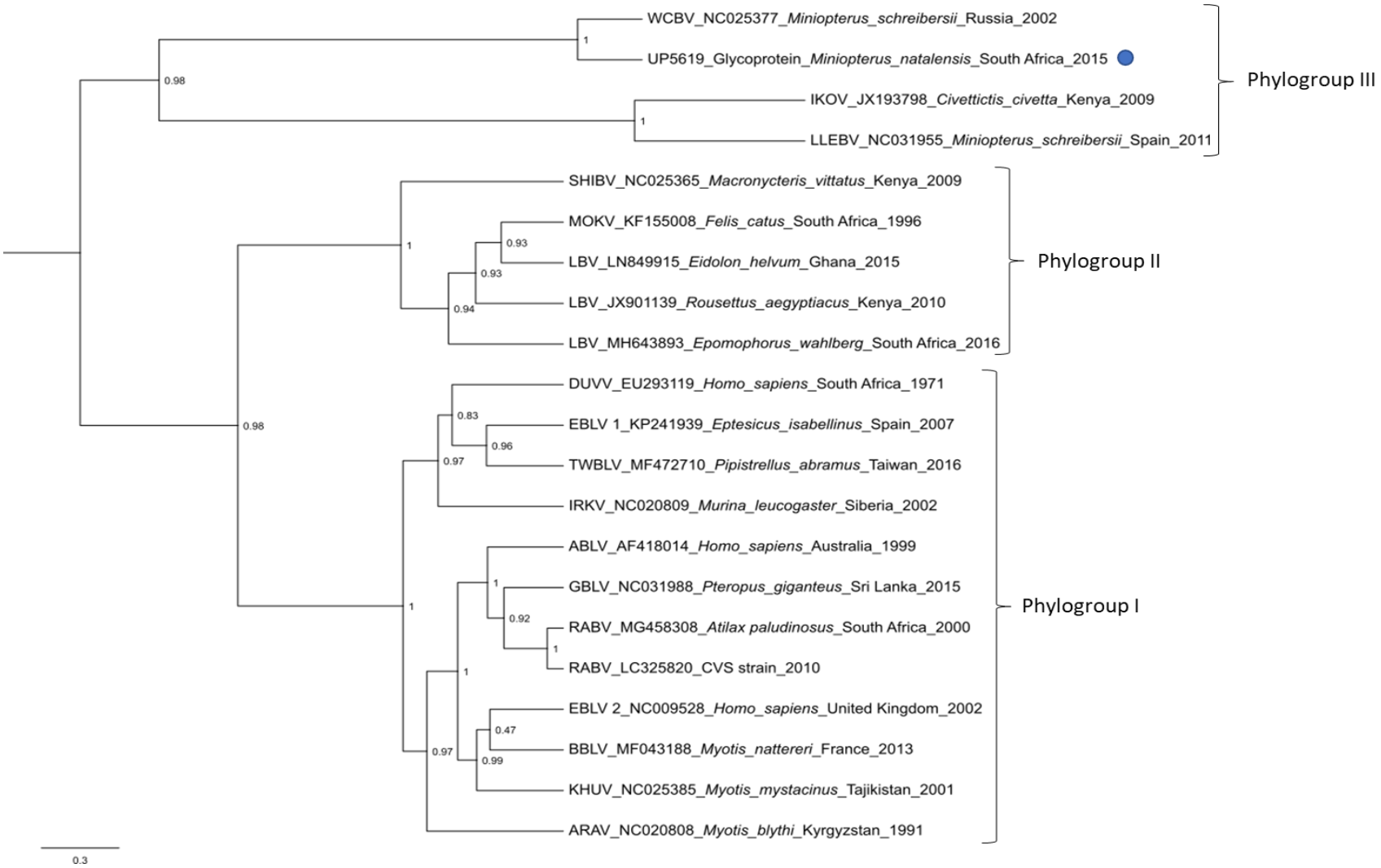


Figure D7 Phylogenetic analyses of the lyssavirus positive sample full G gene sequences generated in this study. Bayesian phylogeny of all concatenated coding regions of the lyssavirus gene sequences was achieved using BEAST employing the GTR+I+G substitution model for posterior probabilities at MCMC chain setting of 10M samples every 1000 states. Lyssavirus positive sample detected in this study are marked with a blue dot.

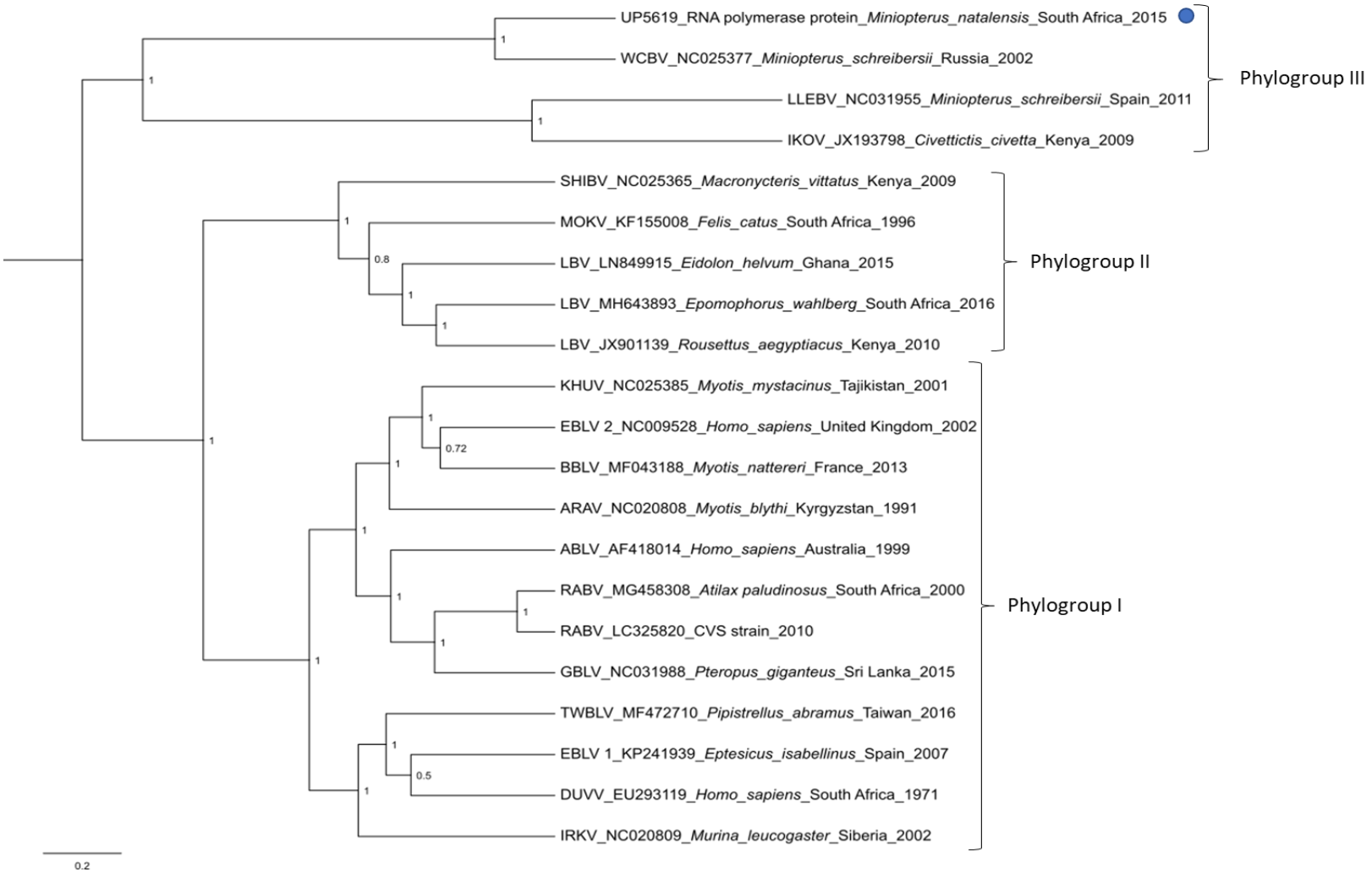


Figure D8 Phylogenetic analyses of the lyssavirus positive sample full L gene sequences generated in this study. Bayesian phylogeny of all concatenated coding regions of the lyssavirus gene sequences was achieved using BEAST employing the GTR+I+G substitution model for posterior probabilities at MCMC chain setting of 10M samples every 1000 states. Lyssavirus positive sample detected in this study are marked with a blue dot.

Appendix E

Table E1 Estimates of evolutionary divergence between lyssavirus N gene sequences when comparing interspecies sequence similarity. The number of base differences per site from between sequences are shown. This analysis involved 18 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 1356 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1-WCBV_NC025377_Miniopterus_schreibersi_Russia_2002																		
2-IKOV_JX193798_Civettictis_civetta_Kenya_2009	0,28																	
3-LLEBV_NC031955_Miniopterus_schreibersii_Spain_2011	0,28	0,25																
4-SHIBV_NC_025365_Hipposideros_commersoni_Kenya_2009	0,26	0,29	0,27															
5-MOKV_KF155008_Feline_South_Africa_1996	0,28	0,30	0,29	0,24														
6-LBV_LN849915_Eidolon_helvum_Ghana_2015	0,25	0,29	0,28	0,23	0,23													
7-ARAV_NC020808_Myotis_blythi_Kyrgyzstan_1991	0,27	0,31	0,30	0,25	0,25	0,24												
8-BBLV_MF043188_Myotis_nattereri_France_2013	0,27	0,31	0,30	0,25	0,27	0,26	0,21											
9-ABLV_AF418014_Homo_sapiens_Australia_1999	0,27	0,32	0,29	0,25	0,27	0,26	0,23	0,21										
10-DUVV_EU293119_Homo_sapien_South_Africa_1971	0,26	0,31	0,29	0,25	0,27	0,27	0,22	0,24	0,23									
11-EBLV_1_KP241939_Eptesicus_isabellinus_Spain_2007	0,28	0,30	0,30	0,26	0,26	0,25	0,22	0,22	0,23	0,21								
12- EBLV_2_NC009528_Homo_sapiens_United_Kingdom_2002	0,27	0,31	0,30	0,27	0,28	0,28	0,23	0,21	0,24	0,24	0,23							
13- GBLV_NC031988_Pteropus_giganteus_Sri_Lanka_2015	0,28	0,32	0,31	0,27	0,27	0,28	0,22	0,23	0,20	0,23	0,22	0,24						
14- IRKV_NC020809_Murina_leucogaster_Siberia_2002	0,28	0,30	0,29	0,25	0,27	0,27	0,24	0,24	0,24	0,22	0,21	0,22	0,25					
15- KHUV_NC025385_Myotis_mystacinus_Tajikistan_2001	0,30	0,31	0,30	0,26	0,27	0,26	0,21	0,19	0,22	0,24	0,23	0,20	0,22	0,24				
16- RABV_LC325820_CVS_strain_2010	0,27	0,31	0,30	0,26	0,26	0,26	0,25	0,24	0,21	0,25	0,25	0,25	0,22	0,26	0,25			
17- KBLV_MF960865_Myotis_sp_Finland_2017	0,28	0,31	0,31	0,27	0,26	0,27	0,20	0,21	0,22	0,24	0,23	0,21	0,22	0,23	0,19	0,24		
18- TWBLV_MF472709_Pipistrellus_abramus_Taiwan_2017	0,27	0,30	0,31	0,25	0,27	0,26	0,23	0,24	0,23	0,22	0,21	0,25	0,24	0,21	0,25	0,25	0,25	

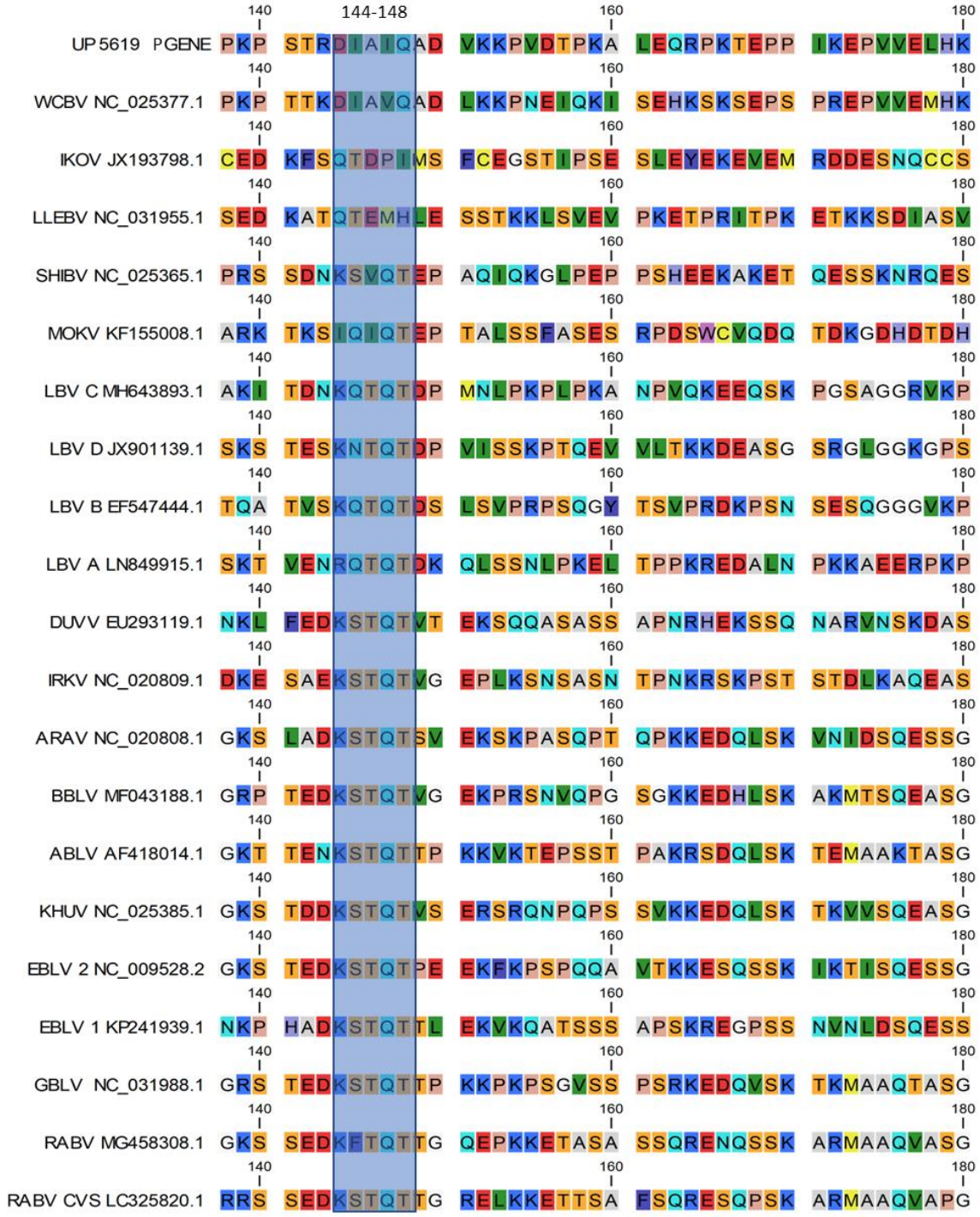
Table E2 Estimates of Evolutionary Divergence between lyssavirus N gene sequences. The number of base differences per site from between sequences are shown. This analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1356 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

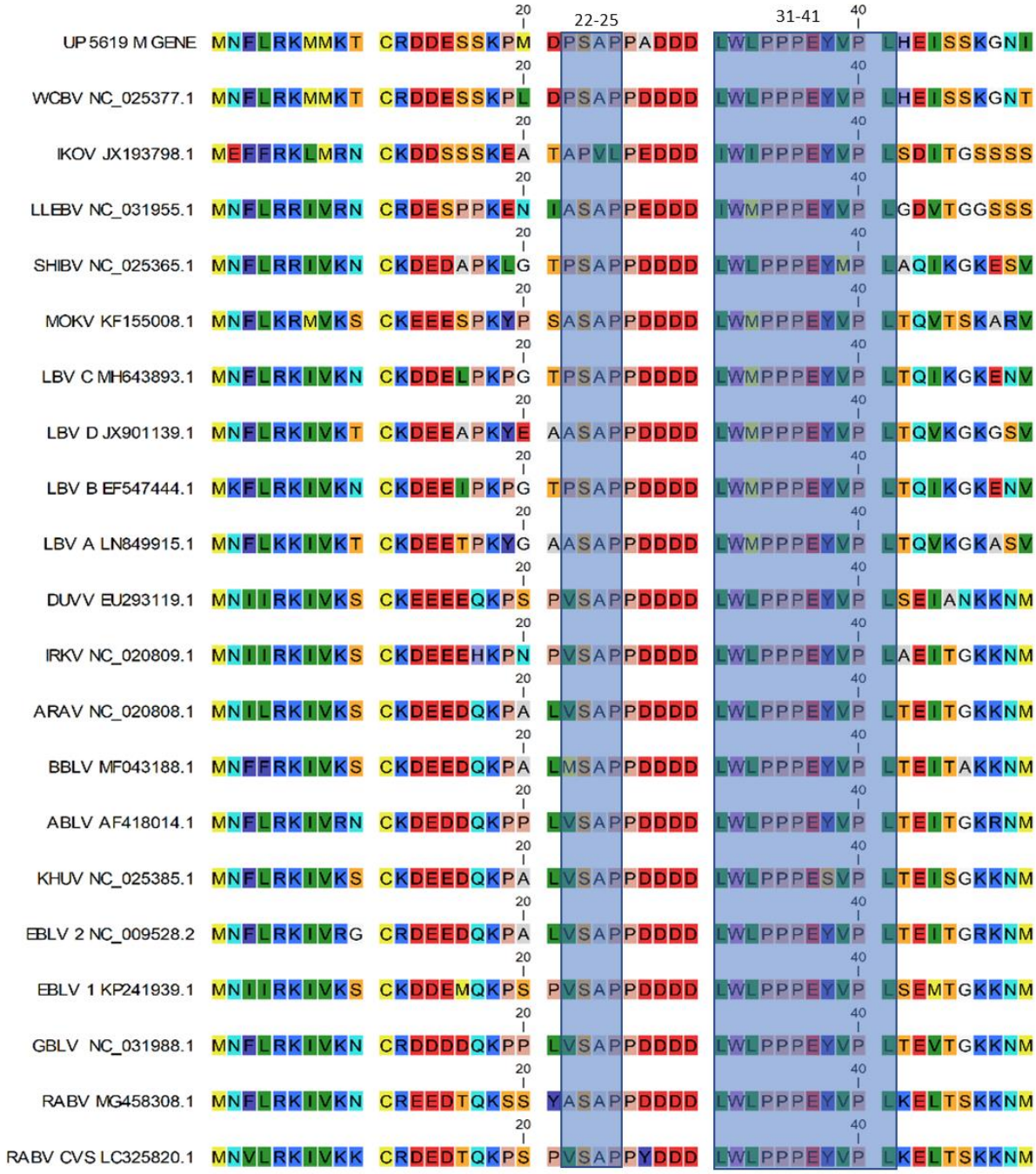
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1- ABLV_NC_003243.1																													
2- ABLV_AY573963.1	0,16																												
3- ABLV_AY573964.1	0,16	0,01																											
4- ABLV_AY573965.1	0,02	0,16	0,16																										
5- DUVV_EU293119.1	0,24	0,22	0,23	0,24																									
6- DUVV_EU623444.1	0,24	0,22	0,23	0,24	0,01																								
7- DUVV_EU293120.1	0,24	0,22	0,23	0,24	0,01	0,01																							
8- DUVV_EU623437.1	0,24	0,22	0,23	0,24	0,00	0,01	0,01																						
9- LBV_A_LN849915.1	0,27	0,26	0,26	0,26	0,27	0,26	0,27	0,27																					
10- LBV_B_EF547459.1	0,27	0,27	0,27	0,27	0,27	0,27	0,27	0,27	0,20																				
11- LBV_D_JX901139.1	0,26	0,24	0,24	0,25	0,25	0,25	0,25	0,25	0,21	0,20																			
12- LBV_C_MH643893.1	0,27	0,25	0,25	0,27	0,25	0,25	0,25	0,25	0,20	0,17	0,19																		
13- LBV_EF547448.1	0,27	0,26	0,26	0,26	0,26	0,26	0,26	0,26	0,01	0,20	0,20	0,20																	
14- LBV_EF547447.1	0,27	0,26	0,26	0,26	0,27	0,26	0,27	0,27	0,01	0,20	0,21	0,20	0,01																
15- LBV_HM179509.1	0,27	0,25	0,25	0,26	0,25	0,25	0,25	0,25	0,20	0,17	0,19	0,00	0,20	0,20															
16- LBV_DQ499948.1	0,27	0,24	0,25	0,26	0,25	0,25	0,25	0,25	0,20	0,17	0,19	0,00	0,20	0,20	0,00														
17- LLEBV_NC_031955.1	0,30	0,28	0,28	0,30	0,29	0,30	0,30	0,29	0,28	0,28	0,29	0,29	0,28	0,28	0,29	0,29													
18- LLEBV_KY006983.1	0,30	0,28	0,28	0,30	0,29	0,30	0,30	0,29	0,28	0,28	0,29	0,29	0,28	0,28	0,29	0,29	0,00												
19- LLEBV MG983927.1	0,30	0,28	0,28	0,30	0,29	0,30	0,30	0,30	0,28	0,28	0,29	0,29	0,28	0,28	0,29	0,29	0,00	0,00											
20- MOKV_KF155008.1	0,27	0,27	0,27	0,27	0,27	0,27	0,27	0,27	0,23	0,23	0,23	0,23	0,23	0,23	0,22	0,22	0,29	0,29	0,29										
21- MOKV_EU293118.1CAR	0,27	0,26	0,26	0,27	0,26	0,26	0,26	0,26	0,24	0,24	0,22	0,22	0,24	0,24	0,22	0,22	0,29	0,29	0,29	0,12									
22- MOKV_KF155006.1ZIM	0,28	0,27	0,27	0,28	0,27	0,26	0,27	0,26	0,24	0,23	0,23	0,22	0,24	0,24	0,22	0,21	0,29	0,29	0,28	0,11	0,12								

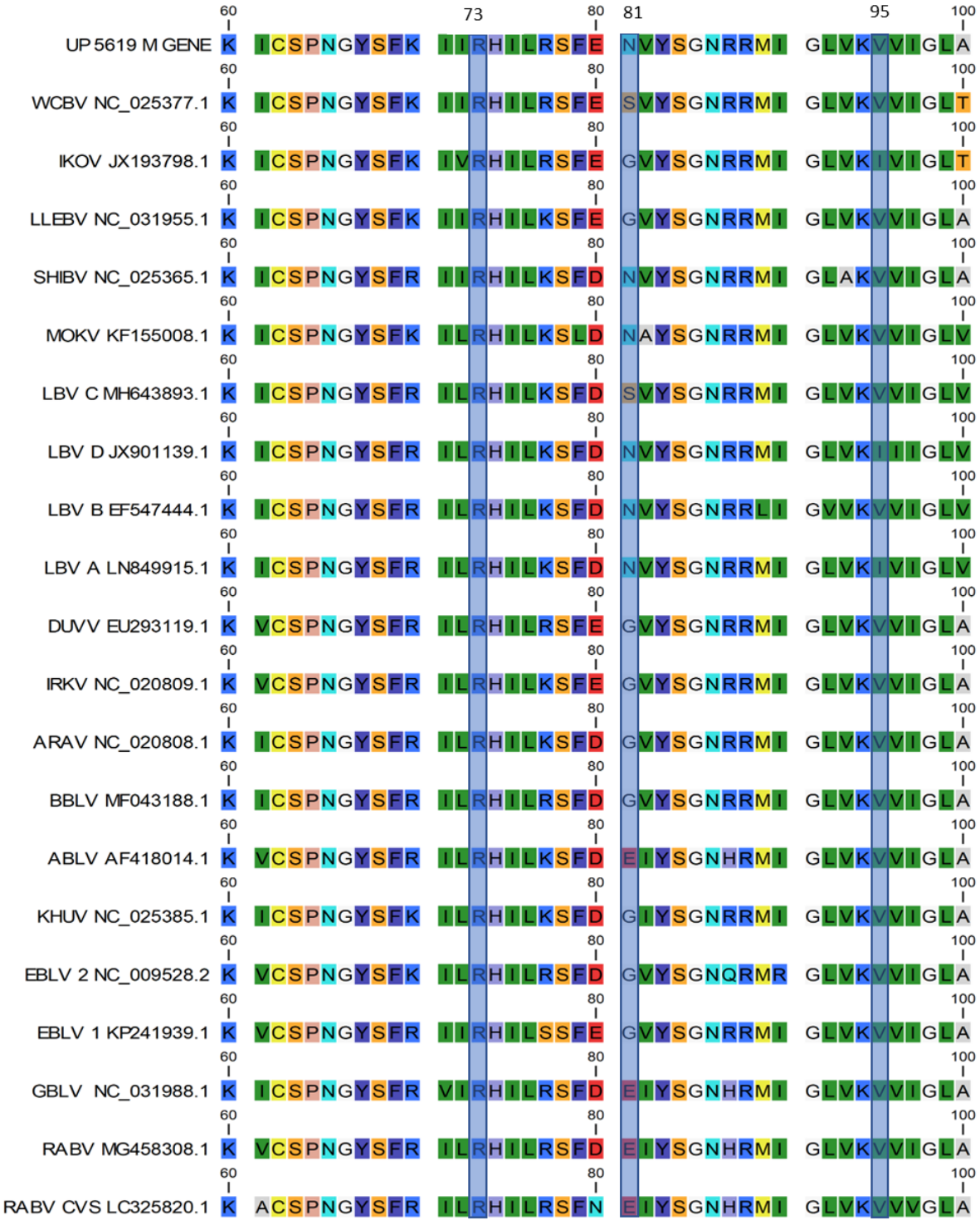
23- MOKV_KF155005.1NIG	0,28	0,28	0,28	0,28	0,27	0,27	0,27	0,27	0,23	0,24	0,23	0,23	0,24	0,24	0,23	0,23	0,30	0,30	0,29	0,12	0,11	0,12							
24- RABV_KT336437.1	0,23	0,21	0,21	0,23	0,26	0,25	0,25	0,26	0,26	0,26	0,26	0,27	0,26	0,26	0,27	0,27	0,30	0,30	0,30	0,27	0,27	0,28	0,27						
25- RABV_MG458308.1	0,25	0,22	0,22	0,24	0,25	0,26	0,26	0,26	0,26	0,27	0,26	0,28	0,26	0,26	0,28	0,27	0,30	0,30	0,30	0,27	0,27	0,27	0,27	0,12					
26- RABV_FJ392391.1	0,24	0,22	0,22	0,24	0,26	0,26	0,26	0,26	0,27	0,27	0,26	0,28	0,28	0,27	0,28	0,28	0,31	0,31	0,31	0,28	0,28	0,28	0,28	0,12	0,09				
27- RABV_AF351847.1	0,23	0,22	0,22	0,23	0,26	0,27	0,26	0,26	0,26	0,26	0,26	0,27	0,26	0,26	0,27	0,27	0,32	0,32	0,32	0,28	0,27	0,28	0,28	0,15	0,16	0,17			
28- RABVCVS_GQ918139.1	0,23	0,21	0,21	0,23	0,25	0,25	0,25	0,25	0,26	0,26	0,26	0,27	0,26	0,26	0,27	0,26	0,30	0,30	0,30	0,26	0,27	0,27	0,27	0,07	0,12	0,12	0,15		
29- RABV_U03770.1	0,24	0,20	0,20	0,24	0,26	0,26	0,26	0,26	0,25	0,27	0,26	0,27	0,25	0,25	0,27	0,27	0,30	0,30	0,30	0,28	0,27	0,27	0,27	0,11	0,13	0,13	0,14	0,11	

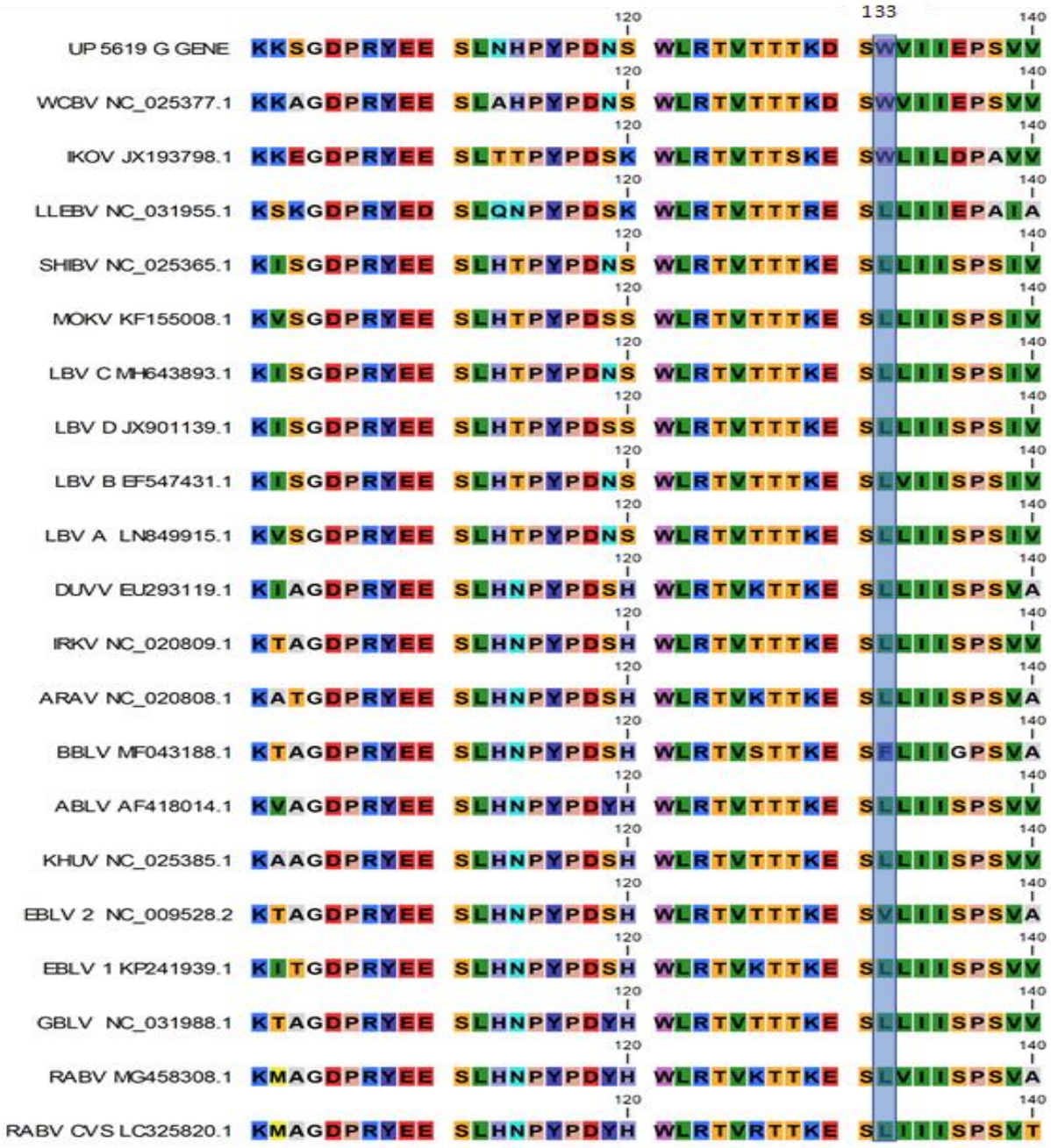
Appendix F

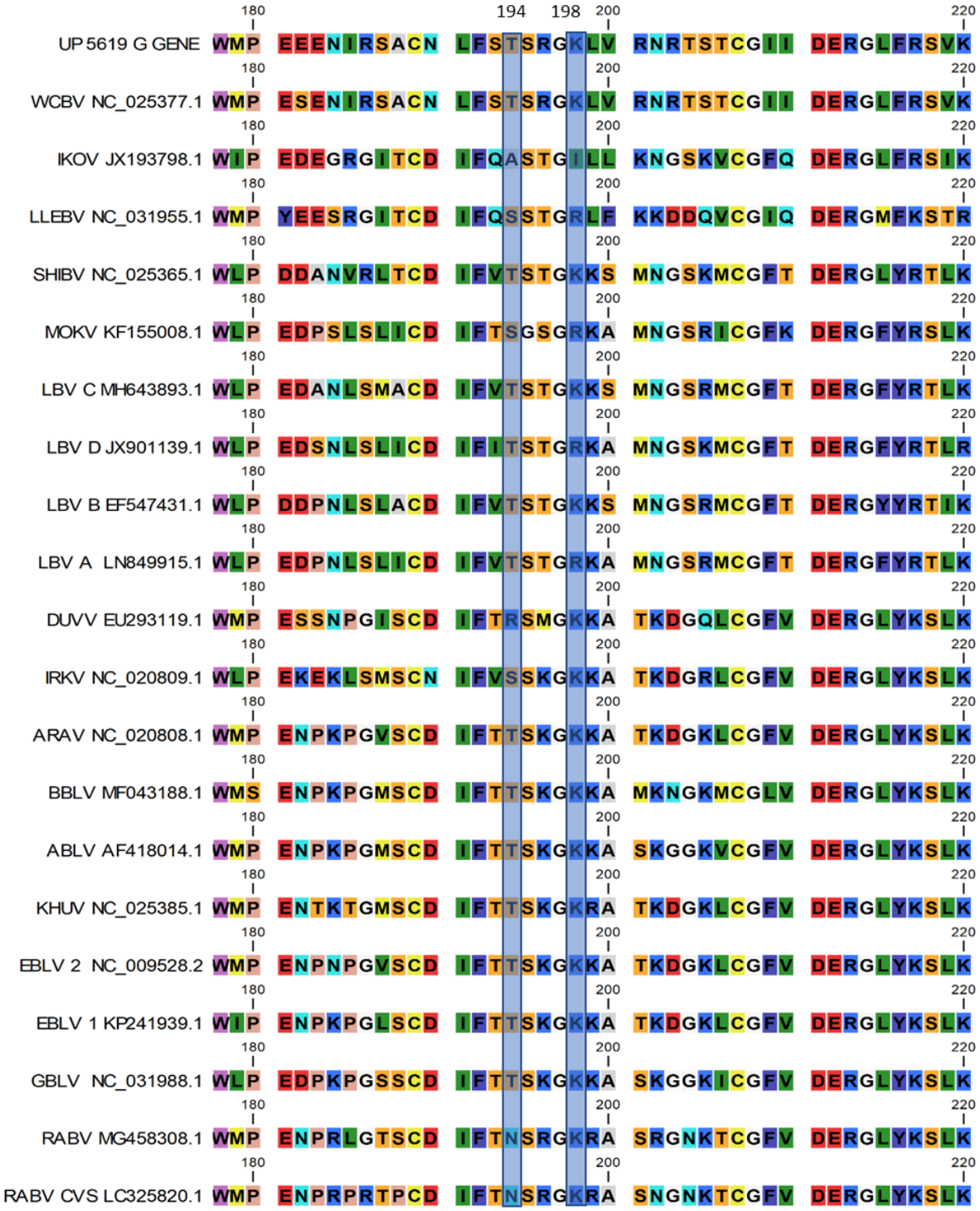
Figure F1 Pathogenic site comparison between MBLV and different lyssavirus species for the P-, M- and G genes.

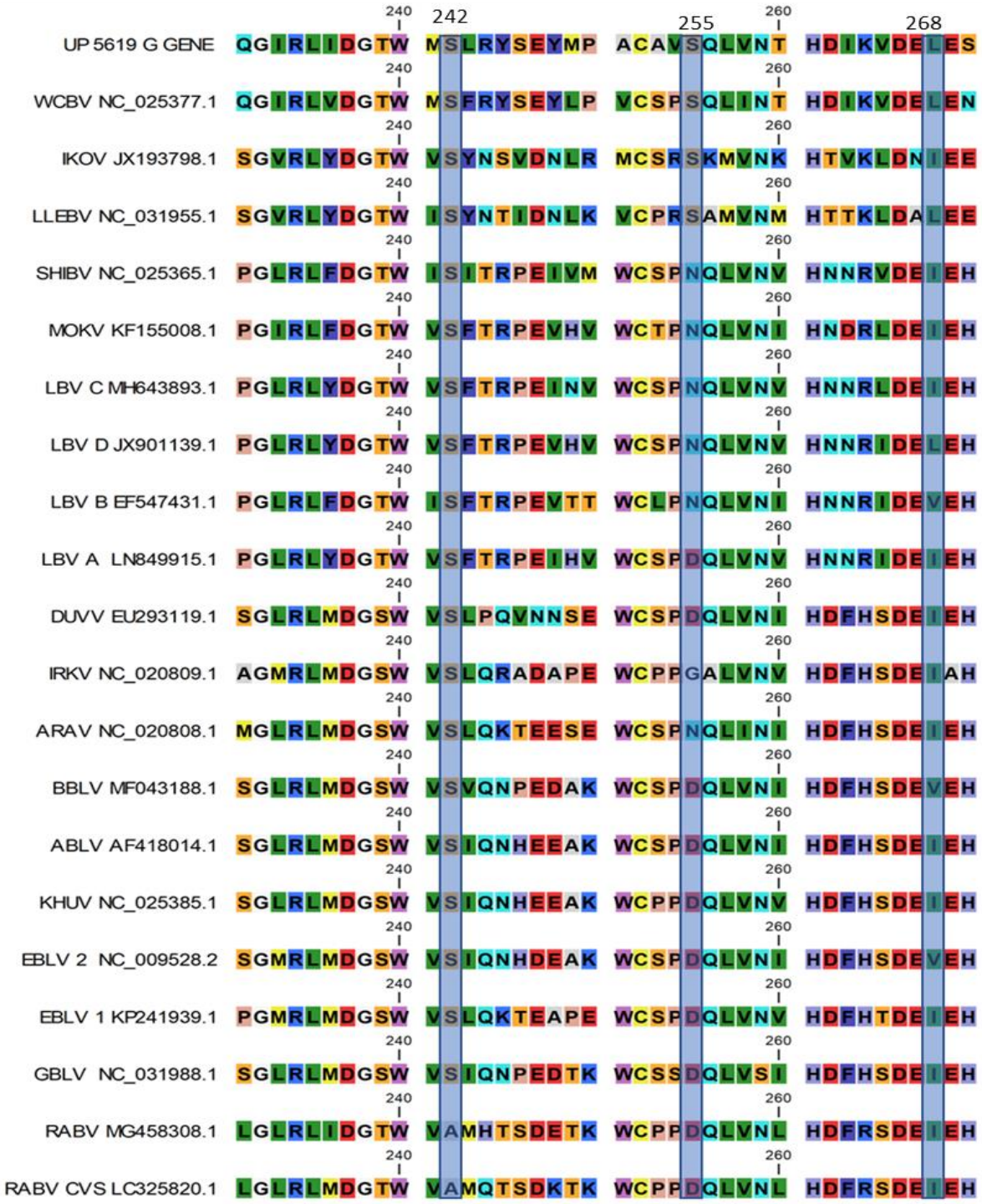


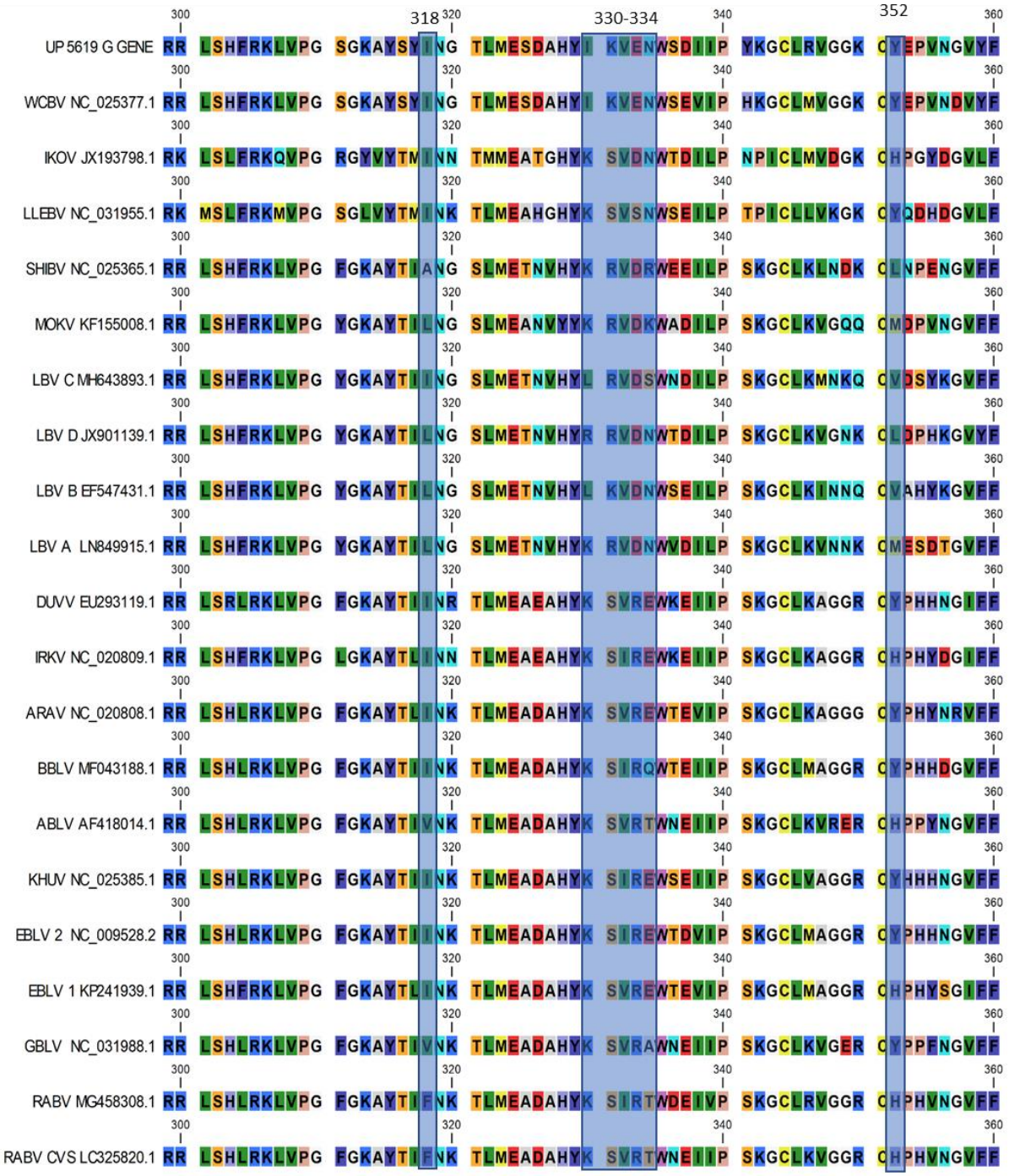

22-25
31-41
UP 5619 M GENE MNFLRKMMKT CRDDESSKPM DPSAPPADDD LWLPPPEYVP LHEISSKGNI
WCBV NC_025377.1 MNFLRKMMKT CRDDESSKPL DPSAPPDDDD LWLPPPEYVP LHEISSKGNT
IKOV JX193798.1 MEFFRKLMRN CKDDSSSKEA TAPVLPEDDD IWIPPPEYVP LSDITGSSSS
LLEBV NC_031955.1 MNFLRRIVRN CRDESPPKEN IASAPPEDDD IWMPPPEYVP LGDVTGGSSS
SHIBV NC_025365.1 MNFLRRIVKN CKDEDAPKLG TPSAPPDDDD LWLPPPEYMP LAQIKGKESV
MOKV KF155008.1 MNFLKRMVKS CKEEESPKYP SASAPPDDDD LWMPPPEYVP LTQVTSKARV
LBV C MH643893.1 MNFLRKIVKN CKDDELPKPG TPSAPPDDDD LWMPPPEYVP LTQIKGKENV
LBV D JX901139.1 MNFLRKIVKT CKDEEAPKYE AASAPPDDDD LWMPPPEYVP LTQVKGKGSV
LBV B EF547444.1 MKFLRKIVKN CKDEEIPKPG TPSAPPDDDD LWMPPPEYVP LTQIKGKENV
LBV A LN849915.1 MNFLKKIVKT CKDEETPKYG AASAPPDDDD LWMPPPEYVP LTQVKGKASV
DUVV EU293119.1 MNIIRKIVKS CKEEEEQKPS PVSAPPDDDD LWLPPPEYVP LSEIANKKNM
IRKV NC_020809.1 MNIIRKIVKS CKDEEEHKPN PVSAPPDDDD LWLPPPEYVP LAEITGKKNM
ARAV NC_020808.1 MNILRKIVKS CKDEEDQKPA LVSAPPDDDD LWLPPPEYVP LTEITGKKNM
BBLV MF043188.1 MNFFRKIVKS CKDEEDQKPA LMSAPPDDDD LWLPPPEYVP LTEITAKKNM
ABLV AF418014.1 MNFLRKIVRN CKDEDDQKPP LVSAPPDDDD LWLPPPEYVP LTEITGKRNM
KHUV NC_025385.1 MNFLRKIVKS CKDEEDQKPA LVSAPPDDDD LWLPPPESVP LTEISGKKNM
EBLV 2 NC_009528.2 MNFLRKIVRG CRDEEDQKPA LVSAPPDDDD LWLPPPEYVP LTEITGRKNM
EBLV 1 KP241939.1 MNIIRKIVKS CKDDEMQKPS PVSAPPDDDD LWLPPPEYVP LSEMTGKKNM
GBLV NC_031988.1 MNFLRKIVKN CRDDDDQKPP LVSAPPDDDD LWLPPPEYVP LTEVTGKKNM
RABV MG458308.1 MNFLRKIVKN CREEDTQKSS YASAPPDDDD LWLPPPEYVP LKELTSKKNM
RABV CVS LC325820.1 MNVLRKIVKK CRDEDTQKPS PVSAPPYDDD LWLPPPEYVP LKELTSKKNM


UP 5619 M GENE
WCBV NC_025377.1
IKOV JX193798.1
LLEBV NC_031955.1
SHIBV NC_025365.1
MOKV KF155008.1
LBV C MH643893.1
LBV D JX901139.1
LBV B EF547444.1
LBV A LN849915.1
DUVV EU293119.1
IRKV NC_020809.1
ARAV NC_020808.1
BBLV MF043188.1
ABLV AF418014.1
KHUV NC_025385.1
EBLV 2 NC_009528.2
EBLV 1 KP241939.1
GBLV NC_031988.1
RABV MG458308.1
RABV CVS LC325820.1


133
UP 5619 G GENE KKSGDPRYEE SLNHPYPDNS WLRTVTTTKD SWVIIEPSVV
WCBV NC_025377.1 KKAGDPRYEE SLAHPYPDNS WLRTVTTTKD SWVIIEPSVV
IKOV JX193798.1 KKEGDPRYEE SLTTPYPDSK WLRTVTTSKE SWLILDPAVV
LLEBV NC_031955.1 KSKGDPRYED SLQNPYPDSK WLRTVTTTRE SLLIIEPAIA
SHIBV NC_025365.1 KISGDPRYEE SLHTPYPDNS WLRTVTTTKE SLLIISPSIV
MOKV KF155008.1 KVSGDPRYEE SLHTPYPDSS WLRTVTTTKE SLLIISPSIV
LBV C MH643893.1 KISGDPRYEE SLHTPYPDNS WLRTVTTTKE SLLIISPSIV
LBV D JX901139.1 KISGDPRYEE SLHTPYPDSS WLRTVTTTKE SLLIISPSIV
LBV B EF547431.1 KISGDPRYEE SLHTPYPDNS WLRTVTTTKE SLVIISPSIV
LBV A LN849915.1 KVSGDPRYEE SLHTPYPDNS WLRTVTTTKE SLLIISPSIV
DUVV EU293119.1 KIAGDPRYEE SLHNPYPDSH WLRTVKTTKE SLLIISPSVA
IRKV NC_020809.1 KTAGDPRYEE SLHNPYPDSH WLRTVTTTKE SLLIISPSVV
ARAV NC_020808.1 KATGDPRYEE SLHNPYPDSH WLRTVKTTKE SLLIISPSVA
BBLV MF043188.1 KTAGDPRYEE SLHNPYPDSH WLRTVSTTKE SFLIIGPSVA
ABLV AF418014.1 KVAGDPRYEE SLHNPYPDYH WLRTVTTTKE SLLIISPSVV
KHUV NC_025385.1 KAAGDPRYEE SLHNPYPDSH WLRTVTTTKE SLLIISPSVV
EBLV 2 NC_009528.2 KTAGDPRYEE SLHNPYPDSH WLRTVTTTKE SVLIISPSVA
EBLV 1 KP241939.1 KITGDPRYEE SLHNPYPDSH WLRTVKTTKE SLLIISPSVV
GBLV NC_031988.1 KTAGDPRYEE SLHNPYPDYH WLRTVTTTKE SLLIISPSVV
RABV MG458308.1 KMAGDPRYEE SLHNPYPDYH WLRTVKTTKE SLVIISPSVA
RABV CVS LC325820.1 KMAGDPRYEE SLHNPYPDYH WLRTVRTTKE SLIIISPSVT


194
198
UP 5619 G GENE WMP EEENIRSACN LFSTSRGKLV RNRTSTCGII DERGLFRSVK
WCBV NC_025377.1 WMP ESENIRSACN LFSTSRGKLV RNRTSTCGII DERGLFRSVK
IKOV JX193798.1 WIP EDEGRGITCD IFQASTGILL KNGSKVCGFQ DERGLFRSIK
LLEBV NC_031955.1 WMP YEESRGITCD IFQSSTGRLF KKDDQVCGIQ DERGMFKSTR
SHIBV NC_025365.1 WLP DDANVRLTCD IFVTSTGKKS MNGSKMCGFT DERGLYRTLK
MOKV KF155008.1 WLP EDPSLSLICD IFTSGSGRKA MNGSRICGFK DERGFYRSLK
LBV C MH643893.1 WLP EDANLSMACD IFVTSTGKKS MNGSRMCGFT DERGFYRTLK
LBV D JX901139.1 WLP EDSNLSLICD IFITSTGRKA MNGSKMCGFT DERGFYRTLR
LBV B EF547431.1 WLP DDPNLSLACD IFVTSTGKKS MNGSRMCGFT DERGYYRTIK
LBV A LN849915.1 WLP EDPNLSLICD IFVTSTGRKA MNGSRMCGFT DERGFYRTLK
DUVV EU293119.1 WMP ESSNPGISCD IFTRSMGKKA TKDGQLCGFV DERGLYKSLK
IRKV NC_020809.1 WLP EKEKLSMSCN IFVSSKGKKA TKDGRLCGFV DERGLYKSLK
ARAV NC_020808.1 WMP ENPKPGVSCD IFTTSKGKKA TKDGKLCGFV DERGLYKSLK
BBLV MF043188.1 WMS ENPKPGMSCD IFTTSKGKKA MKNGKMCGLV DERGLYKSLK
ABLV AF418014.1 WMP ENPKPGMSCD IFTTSKGKKA SKGGKVCGFV DERGLYKSLK
KHUV NC_025385.1 WMP ENTKTGMSCD IFTTSKGKRA TKDGKLCGFV DERGLYKSLK
EBLV 2 NC_009528.2 WMP ENPNPGVSCD IFTTSKGKKA TKDGKLCGFV DERGLYKSLK
EBLV 1 KP241939.1 WIP ENPKPGLSCD IFTTSKGKKA TKDGKLCGFV DERGLYKSLK
GBLV NC_031988.1 WLP EDPKPGSSCD IFTTSKGKKA SKGGKICGFV DERGLYKSLK
RABV MG458308.1 WMP ENPRLGTSCD IFTNSRGKRA SRGNKTCGFV DERGLYKSLK
RABV CVS LC325820.1 WMP ENPRPRTPCD IFTNSRGKRA SNGNKTCGFV DERGLYKSLK


240
242
255
260
268
UP 5619 G GENE QGIRLIDGTW MSLRYSEYMP ACAVSQLVNT HDIKVDELES
WCBV NC_025377.1 QGIRLVDGTW MSFRYSEYLP VCSPSQLINT HDIKVDELEN
IKOV JX193798.1 SGVRLYDGTW VSYNSVDNLR MCSRSKMVNK HTVKLDNIEE
LLEBV NC_031955.1 SGVRLYDGTW ISYNTIDNLK VCPRSAMVNM HTTKLDALEE
SHIBV NC_025365.1 PGLRLFDGTW ISITRPEIVM WCSPNQLVNV HNNRVDEIEH
MOKV KF155008.1 PGIRLFDGTW VSFTRPEVHV WCTPNQLVNI HNDRLDEIEH
LBV C MH643893.1 PGLRLYDGTW VSFTRPEINV WCSPNQLVNV HNNRLDEIEH
LBV D JX901139.1 PGLRLYDGTW VSFTRPEVHV WCSPNQLVNV HNNRIDELEH
LBV B EF547431.1 PGLRLFDGTW ISFTRPEVTT WCLPNQLVNI HNNRIDEVEH
LBV A LN849915.1 PGLRLYDGTW VSFTRPEIHV WCSPDQLVNV HNNRIDEIEH
DUVV EU293119.1 SGLRLMDGSW VSLPQVNNSE WCSPDQLVNI HDFHSDEIEH
IRKV NC_020809.1 AGMRLMDGSW VSLQRADAPE WCPPGALVNV HDFHSDEIAH
ARAV NC_020808.1 MGLRLMDGSW VSLQKTEESE WCSPNQLINI HDFHSDEIEH
BBLV MF043188.1 SGLRLMDGSW VSVQNPEDAK WCSPDQLVNI HDFHSDEVEH
ABLV AF418014.1 SGLRLMDGSW VSIQNHEEAK WCSPDQLVNI HDFHSDEIEH
KHUV NC_025385.1 SGLRLMDGSW VSIQNHEEAK WCPPDQLVNV HDFHSDEIEH
EBLV 2 NC_009528.2 SGMRLMDGSW VSIQNHDEAK WCSPDQLVNI HDFHSDEVEH
EBLV 1 KP241939.1 PGMRLMDGSW VSLQKTEAPE WCSPDQLVNV HDFHTDEIEH
GBLV NC_031988.1 SGLRLMDGSW VSIQNPEDTK WCSSDQLVSI HDFHSDEIEH
RABV MG458308.1 LGLRLIDGTW VAMHTSDETK WCPPDQLVNL HDFRSDEIEH
RABV CVS LC325820.1 LGLRLMDGTW VAMQTSDKTK WCPPDQLVNL HDFRSDEIEH


318
330-334
352
UP 5619 G GENE
WCBV NC_025377.1
IKOV JX193798.1
LLEBV NC_031955.1
SHIBV NC_025365.1
MOKV KF155008.1
LBV C MH643893.1
LBV D JX901139.1
LBV B EF547431.1
LBV A LN849915.1
DUVV EU293119.1
IRKV NC_020809.1
ARAV NC_020808.1
BBLV MF043188.1
ABLV AF418014.1
KHUV NC_025385.1
EBLV 2 NC_009528.2
EBLV 1 KP241939.1
GBLV NC_031988.1
RABV MG458308.1
RABV CVS LC325820.1