Barcoding of South African bat species and evaluation of their natural exposure to lyssaviruses

Stewart Douglas McCulloch

25072570

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae (Microbiology),

In the Faculty of Natural & Agricultural Sciences

University of Pretoria.

Pretoria

Supervisor: Prof. W. Markotter

Co- Supervisor: Prof. L. H. Nel

July 2013

I, Stewart Douglas McCulloch, declare that the thesis/dissertation, which I hereby submit for the degree M.Sc. (Microbiology) at the University of Pretoria, South Africa, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Stewart Douglas McCulloch

30/07/2013

Acknowledgements

I would like to extend my heartfelt thanks to the following:

My supervisors for their guidance, patience, help and feedback in the completion of this manuscript.

The University of Pretoria for giving me this opportunity and for funding.

The National Research foundation of South Africa and Poliomyelitis Research foundation for funding this research.

Dr. Teresa Kearney, curator of small mammals at the Ditsong National Museum of Natural History (formerly the Transvaal Museum) South Africa and Professor Peter Taylor, Department of Environmental Sciences at the University of Venda, South Africa, for their priceless assistance in sample collection and morphological identification.

Professor Wanda Markotter and Professor Louis Nel, in the department of Microbiology and Plant Pathology, University of Pretoria, South Africa, Professor Janusz Paweska, Head of the Centre for Emerging and Zoonotic Pathogens at the National Institute of Communicable Diseases, South Africa, Dr. Jacqueline Weyer, Senior Medical Scientist at the National Institute of Communicable Diseases, South Africa, Dr. Petrus Jansen van Vuren, Medical Scientist at the National Institute of Communicable Diseases, South Africa, Mr Alan Kemp, Senior Medical Scientist at the National Institute of Communicable Diseases, South Africa and Mr Ernest Seamark, Director of African Bats, South Africa, for all their help during sample collection and the field work portions of this research project.

All my colleagues in the Virology laboratories and my friends who have helped and advised me throughout this project.

My parents and family for their on-going support and encouragement during this project.

Summary

The genus *Lyssavirus*, currently consisting of 12 confirmed and two putative species, all of which are capable of producing the lethal encephalitic disease known as rabies. Due to the long history and common knowledge of the prototype virus, rabies virus the other members of this genus, which have only been discovered since the 1960's, are commonly referred to as the rabies-related viruses. Rabies virus is largely maintained throughout the world by on-going viral cycles within non-volant carnivores, whilst the rabies-related members are typically associated with chiropteran populations.

From previous virus isolations and antibody detection, South African bats are host to and come into contact with Lagos bat virus and Duvenhage virus, present within different host populations and are maintained in separate enzootic cycles. Despite limited surveillance for the African continent, these viruses have been found in association with several bat species. Lagos bat virus has been found throughout the continent in association with the old world fruit bats of the *Pteropodidae* family, whilst Duvenhage virus has been found in southern and eastern African in association with smaller insectivorous species with isolations coming from *Nycteris thebaica* and implications with *Miniopterus spp..* The relatively recently described Shimoni bat virus isolated from a Kenyan *Hipposideros vittatus* could follow the Duvenhage distribution through Africa with the distribution of host species. In addition to these three viruses, antibodies have been detected in *Miniopterus spp.* from east Africa, suggesting the presence of West Caucasian bat virus however the other members of the bat-associate lyssaviruses are as yet to be isolated or implicated with Africa. Conversely, rabies virus, Mokola virus and Ikoma lyssavirus

are present on the African continent but have not been detected within African bat populations.

Serological analysis of 324 South African bat samples was performed to demonstrate the presence of antibodies capable of neutralising; Duvenhage virus, Lagos bat virus and rabies virus. This analysis is the first step in delimiting bat species that come into contact with these viruses and provides an understanding of the level of contact in the meta-population of bats. From this serology of 28 bat species it was seen that, 33 % of samples were able to neutralise Lagos bat virus, whilst 13 % were capable of neutralising Duvenhage virus and interestingly two percent had antibodies able to neutralise rabies virus. Brain tissues of 413 bats were tested for the presence of lyssavirus genomic RNA. One of these samples tested positive, a *Nycteris thebaica* from Limpopo, provided a new isolation of Duvenhage virus.

Tissue samples of 117 morphologically identified bats collected throughout South Africa were used for the DNA barcoding of their genomes via the use of the mitochondrial cytochrome *c* oxidase subunit 1 (COI). These samples provided sequence for 27 species of bats from 18 genera, representing the first COI sequences for South African bats and providing the first COI sequences available for many of the species. With compilation into phylogenetic trees, these sequences demonstrate the use of COI as an identification tool and form the basis of a COI database of South African bat species, to which future samples may be compared with to obtain phylogenetic identifications, ultimately aiding in the identification of host species of the diseases.

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List of abbreviations

µg – microgram
µl – microlitre
ABLV – Australian bat lyssavirus
AMV – avian myeloblastosis virus
ARAV – Aravan virus
BBLV – Bokeloh bat lyssavirus
B.C. – Before Christ
BOLD – Barcode of Life Database
bp – base pairs
C – Celsius
cDNA – complimentary deoxyribose nucleic acid
CNS – central nervous system
COI – mitochondrial cytochrome oxidase subunit 1
CO_2 – carbon dioxide
CVS – challenge virus standard
DFA – direct fluorescent antibody
DMEM – Dulbeccos Modified Eagle Medium
DNA – deoxyribose nucleic acid
dNTPs – deoxyribonucleotide triphosphates
DUVV – Duvenhage virus
EBLV1/2 – European bat lyssavirus 1/2
EDTA – ethylene diamine tetra acetic acid
ELISA – Enzyme-Linked Immunosorbent Assay
EtBr – ethidium bromide
FAT – Fluorescent antibody test
FAVN – Fluorescent Antibody Virus Neutralization
FITC – Fluorescein isothyocyanate
g – gravity
G – Glycoprotein
ICTV – International Committee for the Taxonomy of Viruses
IRKV – Irkut virus
IKOV – Ikoma lyssavirus
KCl – potassium chloride
KHUV – Khujand virus
km – kilometers
L – Polymerase protein
LBV – Lagos bat virus
M – Matrix protein
mg – milligram
$MgCl_2$ – magnesium chloride
ml – millilitre
mm – millimetre
mM – millimolar
MNA – mouse neuroblastoma
MOKV – Mokola virus
MYA – million years ago
N – Nucleoprotein
NJ – Neighbor-joining
nm – nanometer
nt – nucleotide
P – Phosphoprotein
PBS – phosphate buffered saline
PCR – polymerase chain reaction
pmol – picomol
qRT – quantative real-time
RABV – rabies virus
RFFIT – Rapid Fluorescent Focus Inhibition Test
RFU – relative fluorescence units

RNA – ribonucleic acid
RT-PCR – reverse-transcription polymerase chain reaction
SA – South Africa
SHIBV – Shimoni bat lyssavirus
Taq – *Thermus aquaticus*
TCID – tissue culture infective dose
Tris-HCl – Tris-Hydrochloride
U – units
UV – ultra violet
v – volts
WCBV – West Caucasian bat virus
WHO – World Health Organisation
w/v – weight per volume

Chapter 1

Literature Review

1.1. Introduction

Over the past 60 years the genus *Lyssavirus* has expanded to contain 12 confirmed and two putative species. Within the genus Rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), Shimoni bat virus (SHIBV) and the putative species of Ikoma lyssavirus (IKOV) have all been isolated from Africa, whilst antibodies capable of neutralising West Caucasian bat virus (WCBV) have been identified within Kenyan bat populations (Kuzmin, *et al.,* 2008b; Kuzmin, *et al.,* 2010). Of these seven viruses, DUVV and the members of the phylogroup II; LBV, MOKV, SHIBV and the new IKOV are unique to the continent. LBV, DUVV, WCBV and SHIBV have all been demonstrated to be associated with bat populations as their most likely reservoir hosts, with occasional 'spill-over' to other vertebrates (Delmas, *et al.,* 2008; Kuzmin, *et al.,* 2010; Markotter, *et al.,* 2008b). As described by Turmelle and Olival, (2010) there are several contributing factors as to why bats serve as such interesting and unique hosts for these viruses. Some of the most relevant reasons are: (1) when compared to other mammals of similar size and weight, the bat is prone to longevity with life spans stretching into decades, (2) bats typically form colonies some of which can amass to the hundreds of thousands, which equates to ample individuals to be infected by the spreading virus, (3) within these colonies the individuals are very social, with significant amounts of body contact, social grooming and food sharing which all provide opportunities for transmission

between individuals and (4) due to their ability of flight, bats are able to traverse huge distances fairly rapidly, dramatically increasing the transmissible range of the virus and providing opportunities for the mixing of different populations.

This project was designed to advance the knowledge of lyssaviruses in South Africa which occur within native bat populations, and how these South African bat associated lyssaviruses are maintained and spread within these populations. This study began with the process of identifying which species of southern African bats have been exposed to lyssaviruses. Therefore blood sera of sampled bats were analysed for neutralizing antibodies against the African associated lyssaviruses LBV and DUVV as well as RABV. A further aim was to detect and isolate any lyssavirus present in the brain tissues of available bat samples.

In addition to the virological work, DNA barcoding sequences of morphologically identified South African bat species were produced. This work produced the first basic COI sequence database for South African bats, which should be expanded upon in future studies. The utility of such a database is that new samples may be identified (on a species level) in a fast and accurate manner, based on a comparison with the sequence database. Such genetic identification is likely to play a vital role in future and, apart from accuracy and speed, also negates the need to have the entire carcass (as is the case for morphological identification).

For the purposes of this study an overview of the available information will be provided in the following literature review. The overview divides the work into two general sections; (A) Information available about the

lyssaviruses, with specific focus on those viruses associated with Africa and African bat populations. (B) A general overview of chiroptera, specifically South African bats and a summary of DNA barcoding and its application to bats.

A. Lyssaviruses

1.2. Overview of Lyssaviruses

1.2.1. Taxonomy and diversity

The genus *Lyssavirus* belongs to the family of *Rhabdoviridae* within the order *Mononegavirales* (all the single stranded, negative sense, non-segmented RNA genome viruses; ICTV, 2011). Among the rhabdoviruses, the vast majority are associated with plant and invertebrate infections. The genus *Lyssavirus* represents one of the most important members of the family with regards to public and veterinary health and the associated zoonosis. After the initial onset of clinical symptoms it is most common for a lyssavirus infection to progress to a state of acute encephalitis with a 100 % fatality (Johnson, *et al.,* 2010). The lyssaviruses currently contains 12 confirmed and two putative member species of virus (ICTV, 2011). All members of this genus have the ability to cause lethal encephalitis in mammals, including humans.

The disease caused by these viral infections is named after the initial member of the group, rabies virus (RABV). Since the second half of the 20th century with the discovery of Lagos bat virus (LBV) and Mokola virus (MOKV) the group of so called rabies-related viruses, (viruses which share the physical characteristics and are genetically related to rabies virus yet can be distinguished by their antigenic and genetic differences), has been steadily increasing.

1.2.1.1. Species recognised

The information available for this genus has been rapidly increasing since 1956 when it was first discovered that the type specific (RABV) was not

the sole member of this group. This was realised after, a newly identified rabies variant found in a Nigerian fruit bat (*Eidolon helvum*), was classified as LBV and became the first rabies-related virus to be included into the genus *Lyssavirus* (Boulger, 1958; Shope, *et al*. 1970). This genus of viruses was originally divided into sero-groups depending on their reaction with monoclonal antibodies, with those able to be detected by the same antibodies being grouped together (Dietzschold, *et al.* 1988; King, *et al*., 1993). Then with the development of molecular techniques the sero group terminology was replaced with genotype, giving rise to first six members of the genus, representing the differences between the sero groups on a genetic level (Bourhy, *et al.* 1993; Dietzschold, *et al.* 1988).

Since this initial discovery, there have been multiple species added, bringing the genus to 12 members with two additional putative members awaiting acceptance (ICTV, 2011; Marston, *et al*., 2012b).These species are further separated into three phylogroups, depending upon their phylogenetic diversity and their serological identity. The species that are closer genetically tend to produce overlapping serological results. The first and largest of these phylogroups is phylogroup I made up of rabies virus (RABV), Duvenhage (DUVV), European bat lyssavirus type 1 and type 2 (EBLV-1 and EBLV-2), Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV) and the newly described putative species of Bokeloh bat lyssavirus (BBLV) (Freuling, *et al.,* 2011; Marston, *et al.,* 2012b). Phylogroup II, which is made up of the previously termed genotype two virus Lagos bat virus (LBV), the former genotype three, Mokola virus (MOKV) and the newly described Shimoni bat virus (SHIBV), all of which are uniquely

African viruses. The final phylogroup is more a group of exclusion, being made up of viruses that do not fit the criteria for either of the other groups. West Caucasian bat virus (WCBV) was originally the sole member of this group until the recently described putative Ikoma lyssavirus (IKOV) demonstrated its divergence from all the other members of the genus. (Badrane, *et al.,* 2001; Kuzmin, *et al.,* 2010; Marston, *et al.,* 2012b; Marston, *et al.,* 2012c).

This differentiation is most commonly attributed to variations in the 'N' gene, but the phylogenetic tree can also be constructed according to differences in the 'G' gene, alternatively variations between sequences of N+P+M+G+L concatenations or full genomes where possible, with the same overall shape and grouping being displayed (Kuzmin, *et al.,* 2010).

All the lyssavirus species have been found in association with bat populations throughout the world with the exceptions of MOKV, originally isolated from the organs of a shrew (*Crocidura sp*.) during 1968 (Shope, *et al*., 1970) and the new putative IKOV isolated from an African civet (*Civettictis civetta*) (Marston, *et al*., 2012a). The reservoir hosts of both these exceptions are still unknown.

Table 1.1. Species of lyssavirus obtained to date, including geographical area and animal host the virus was isolated from.

Lyssavirus specie	**Species isolated from**	**Geographical location**
Rabies virus (type specie)	Insectivorous and hematophagic American bats and non-volant carnivores	The Americas world wide
Lagos bat virus	Frugivorous bats (*Epomophorus gambianus, E. wahlbergi, Eidolon helvum, Nycteris gambiensis* and *Rousettus aegyptiacus*)	Africa
Mokola virus #	Originally isolated from shrews (*Crocidura spp.)*	Central and southern Africa
Duvenhage virus	Insectivorous bats (*Nycteris thebaica*)	Sub-Saharan Africa
European bat lyssavirus 1	Insectivorous bats (*Eptesicus serotinus*)	Western Europe
European bat lyssavirus 2	Insectivorous bats (*Myotis daubentonii, M. dasycneme*)	Western Europe
Australian bat lyssavirus	Frugivorous and insectivorous Australian bats	Australia
Aravan virus	Insectivorous bats (*Myotis blythi)*	Eurasia
Khujand virus	Insectivorous bats (*Myotis mystacinus*)	Central Asia
Irkut virus	Insectivorous bats (*Murina leucogaster*)	Eastern Siberia and China
West Caucasian bat virus	Insectivorous bats (*Miniopterus schreibersi*)	Caucasian region
Shimoni bat virus	Insectivorous bats (*Hipposideros vittatus*)	East Africa (Kenya)
Bokeloh bat lyssavirus*	Insectivorous bats (*Myotis nattereri*)	Western Europe (Germany)
Ikoma lyssavirus* #	Isolated from African civet (*Civettictis civetta*)	Eastern Africa (Tanzania)

* represents a putative specie, # indicates that the virus has as yet not been identified within bat specie

1.2.1.2. Diversity

The viral genome is typically well conserved across the different species of the genus. In general, the level of amino acid sequence conservation descending from the nucleoprotein, to the polymerase protein, to the matrix protein, then to the glycoprotein and finally ending with the lowest level of amino acid conservation in the phosphoprotein (Bourhy 1993; Delmas, *et al.,* 2008). In the mid 1990's it was shown that the N-gene similarities between the genotypes known at the time, were less than 79.8 % on a genetic level and less than 93.3 % on amino acid level (Kissi, *et al.,* 1995). From this the ICTV has set a threshold of between 80 and 82 % N-gene nucleotide identity being necessary for the delimitation of species in this genus. Later on, it has been suggested that in addition to this genetic criteria, the involvement of specific host and geographical distribution be brought into consideration for the purposes of species classification.

1.2.2. Viral structure

As was described by Tordo *et al*., 1986 for RABV, the viral particle of all the other *Lyssavirus* genera comply with convention of the *Rhabdoviridae* family. Virus particles are a bullet or rod shaped protein structure with an average diameter of approximately 75 nm and a variable length of between 100 nm and 300 nm. This lipid bilayer with inserted proteins (particularly G) forms one of the two major structural components of each viroid and surrounds the second component, the helical ribonucleoprotein core (RNP). The RNP consists of a non-segmented RNA viral genome and three viral proteins. The lyssavirus genome is typically around 12 000 nucleotides (nt) in

length and encodes five viral proteins. These proteins are encoded in the following order along the 3' to 5' linear RNA strand, with the respective gene sizes, however there is variation amongst the species; nucleoprotein (N) 1353-1356 nt, phosphoprotein (P) 894-918 nt, matrix protein (M) 609 nt, glycoprotein (G) 1569-1602 nt and polymerase protein (L) 6381-6429 nt (Delmas, *et al.,* 2008). Each of these viral proteins has a unique and essential function.

The N protein tightly encases the RNA within the RNP and regulates the transfer from transcription to translation depending on the ratio of protein to viral RNA. The N mRNA and the translated protein are the most abundant of the viral gene products within an infected cell and with its high level of conservation makes it the ideal target for an assay detecting the presence of lyssaviruses. The P forms another portion of the essential RNP whilst the M holds the RNP and viral membrane together in the completed viroid. The G forms the surface protein of the virus as an array of around 400 trimeric spikes. These spikes interact with cell surface receptors during the initial stages of infection. Finally the L which is the viral encoded polymerase both forms the final portion of the RNP and facilitates the transcription of the viral genomic RNA to messenger RNA that can then be translated into the viral proteins.

1.2.3. Replication and pathogenesis

The replication cycle is initiated by the lyssavirus G protein binding to cellular receptors. However the specific receptors targeted are as yet unidentified with RABV demonstrating the ability to affect most cell lines *in vitro*, whilst displaying high levels of neuronal cell tropism *in vivo* (Seganti, *et al.,* 1990). The G protein then mediates the entrance of the virus particle into the host cell via fusion of viral envelope and cell membrane (Superti, *et al.,* 1984). After the internalisation of the virus to the host cytoplasm, viral replication is initiated. The level of N available facilitates the switch from transcription to replication and once there is sufficient accumulation of viral components assembly begins. Viral genomic RNA is encapsulated by the N, P and L proteins, followed by the M protein binding to the RNP complex and producing the characteristic bullet shape. The virus particle then migrates to the cellular membrane where the G protein trimeric spikes are positioned and bind with the M proteins as the virus buds from the host cell acquiring a new lipid envelope.

Lyssavirus infections target the nervous tissues, with retrograde transport along neuronal axons facilitating the movement of the sub-viral particles to the central nervous system at between 12 and 100 mm per day (Tsiang, *et al.,* 1991). This viral spread carries on along the spinal cord to the brain. The virus then rapidly disseminates along terminal axons to salivary glands and epithelial tissue for host to host transmission and is deposited throughout the body of the affected animal (Charlton, 1994; Jackson, 2002).

The period from initial infection till the presentation of clinical symptoms typically takes between two and 12 weeks but this period of incubation may take much longer (Hemachudha, 1994). During the infection of the nervous system, the virus is protected from major histocompatibility class 1 and 2 presenting cells and T lymphocytes due the blood brain barrier preventing passage of these antibodies, lymphocytes and complement proteins (Lafon, 2013). The presence of virus neutralising antibodies is facilitated by an immune response to the G protein but the mode of action is still unclear as the blood brain barrier does not permit the passage of antibodies, prompting the hypothesis that these neutralising antibodies act before the virus enters the neurons. With human infections, neutralising antibodies will not present until late in the clinical phase, by which point the virus has spread to other organs and is facilitating multiple organ failure. The presence of lyssavirus specific neutralising antibodies in apparently unvaccinated, healthy animals indicates that at some point the animal has been exposed to an abortive lyssavirus infection, or that the host has mounted a successful defence against the virus, the reasons for these unsuccessful infections or the mechanisms of the successful response are however still unclear.

1.2.4. Global distribution

The genus of lyssaviruses has representatives on every continent with the exception of Antarctica and some small isolated areas, typically islands, (Nadin-Davis, *et al.,* 2012). Of all the viral members, the prototype virus for this genus, RABV, is the most widely distributed of the known viruses, typically being found within a variety of non-volant carnivores and new world

(the Americas) bat species. RABV is found on all the lyssavirus affected continents except for Australia. In addition to the aforementioned lyssavirus free areas, some developed countries have undertaken extensive vaccination campaigns of non-volant carnivores to eradicate the virus and obtain a ‘rabies free’ status. These campaigns have however excluded the rabies-related viruses and have been unable to address any of the bat associated viruses, focusing purely on rabies virus affected non-volant carnivores. This has left these ’rabies free’ countries susceptible to chiropteran mediated infections and spill-over events. The other members of the genus have to date, appeared to be far more restrictive in their distribution, typically being limited to a single continent and to their hosts with only occasional spill over events to non-volant mammals. It must however be stated that there is a severe lack of surveillance and limited knowledge available for these viruses with the focus typically being on rabies virus.

1.2.5. Lyssaviruses on the African continent

The African continent plays host to six species of lyssavirus namely; RABV, DUVV, LBV, MOKV, SHIBV and the putative IKOV. Additionally to these isolations, antibodies capable of neutralising the phylogroup III member isolated from Europe, WCBV have been identified in *Miniopterus ssp.* in Kenya (Kuzmin, *et al.,* 2008b). Although both the canid and mongoose biotypes of RABV circulate in Africa it has not been isolated in any African bat populations, and the reservoir population of MOKV is as yet undetermined and not believed to be present in bats (Delmas, *et al.,* 2008; Kuzmin, *et al.,* 2010; Markotter, *et al.,* 2006). The new putative IKOV, also present on the African continent portrays a different picture, and although it was isolated from an African civet (*Civettictis civetta*) (Marston, *et al.,* 2012a), it is plausible that this is a bat-associated virus that has as yet avoided detection. This hypothesis exists due to the facts that civets tend to occupy territory in close proximity to cave dwelling bats and despite civets having been under previous surveillance for RABV, IKOV had not been detected before (Sabeta, *et al.,* 2008).

1.2.5.1. Lagos bat virus

The initial isolation of LBV was achieved from pooled brain samples of Nigerian fruit bats (*Eidolon helvum*) during 1956. The animals were collected under the presumption that they were infected with RABV. It was only after the lack of observation of inclusion negri bodies and the reduced pathogenic results in suckling mice, that it was realised that this viral infection was similar to, but most definitely not RABV (Boulger, & Porterfield, 1958; Shope, *et al.,* 1970). It was later confirmed that this new virus although similar to rabies virus in appearance was significantly different and thus would be classified as a rabies-related virus. With the addition of MOKV during 1968 the initial three members of the lyssaviruses formed the newly classified genus (Boulger, & Porterfield, 1958; Shope, *et al.,* 1970). Subsequent to the initial discovery, LBV has been isolated on twenty three occasions, from both non-volant and chiropteran species, (Table 1.2.).

All of the available sequences of LBV are distinguishable as members of this specie; however, there is significant variation within the group. This variation causes the formation of four lineages (lineage; A, B, C and D) within the species, clustering in accordance to the geographical region of isolation (Delmas, *et al.,* 2008; Kuzmin, *et al.,* 2010; Markotter, *et al.,* 2008a). These LBV lineages represent genetic divergence within the species that may in fact show that the current LBV species is a collection of multiple viral species. This congregation of viral divergence may become more evident as the complete extent of the possible reservoir species becomes evident, with the possibility of these lineages diverging from one another as they adapt to their specific host population and its geographical restraints. In addition to these viral isolations there has also been the findings of LBV neutralising antibodies throughout Africa in multiple species of old world fruit bat (*Pteropodidae*), including but not limited to *Eidolon helvum, Epomophorus buettikoferi, Ep. gambianus, Ep. wahlbergi, Nycteris cambiensis* and *Rousettus aegyptiacus* with seroprevelance ranging from 40 to 67 % for *E. helvum* and 29 to 46 % for *R. aegyptiacus.* (Hayman, *et al.,* 2008; Kuzmin, *et al.,* 2010; Markotter, *et al.,* 2006).

Despite the observed presence of high seropositivity within these bat populations and the recorded instances of non-volant spill over infections the current vaccines only provide protection for the phylogroup I viruses, with no coverage being extended to LBV nor the other lyssavirus members of phylogroup II and III (Nel, 2005).

Table 1.2. Bat species from which Lagos bat virus have been isolated, including lineage with the corresponding year and country of isolations to date.

Bat Specie	Year of isolation	Country	LBV lineage	Reference
Epomophorus wahlbergi	2013	South Africa	C	(W. Markotter) Personal correspondence
Rousettus aegyptiacus	2010	Kenya	D	Kuzmin, personal correspondence
Rousettus aegyptiacus	2009	Kenya	D	Kuzmin, personal correspondence
Rousettus aegyptiacus	2008	Kenya	D	Kuzmin *et al.,* 2010
Ep. wahlbergi	2008	South Africa	C	Markotter, personal correspondence
Eidolon. Helvum	2007	Kenya	A	Kuzmin *et al.,* 2008b, 2010
E. helvum	2006-7	Kenya	A	Kuzmin *et al.,* 2008
Ep. Wahlbergi	2006	South Africa	C	Markotter 2007
Ep. Wahlbergi	2005	South Africa	C	Markotter *et al.,* 2006b
Ep. Wahlbergi	2004	South Africa	C	Markotter *et al.,* 2006b
Atilax paludinosus	2004	South Africa	C	Markotter *et al.,* 2006a
Ep. Wahlbergi	2003	South Africa	C	Markotter *et al.,* 2006b
Canis familiaris (dog)	2003	South Africa	C	Markotter *et al.,* 2006b
R. aegyptiacus	1999	Togo/Egypt	A	Aubert 1999
C. familiaris (dog)	1989-90	Ethiopia	C	Mebatsion *et al.,* 1992
Ep. Wahlbergi	1990	South Africa	C	Swanepoel 1993
Felis catus (cat)	1986	Zimbabwe	C	King & Crick 1988
E. helvum	1985	Senegal	A	Swanepoel, 1993
*Nycteris gambiensis**	1985	Guinea	Unknown	Swanepoel, 1993
Felis catus (cat)*	1982	South Africa	C	King & Crick 1988
Ep. Wahlbergi	1980	South Africa	C	King & Crick 1988
Micropteropus pusillus	1974	Central African Republic	C	Sureau *et al.,* 1977
*E. helvum**	1956	Nigeria	B	Boulger & Porterfield 1958

* indicates isolations from more than one individual

1.2.5.2. Duvenhage virus

DUVV was initially discovered in 1970 from the fatal rabies infection of a man in the Bella Bella (formally Warmbaths) area, of the Limpopo province, South Africa, after being bitten by a small insectivorous bat on a farm (Meredith, *et al.,* 1971). The specific bat was never positively identified in this case, but *Miniopterus schreibersii* (now *M. natalensis*) was implicated due to its presence in the area (van der Merwe, 1982). DUVV was again isolated during 1981 in South Africa, from the carcass of a small insectivorous bat (the species was never verified), that was caught by a cat during the day in the Louis Trichardt area of South Africa (Paweska, *et al.,* 2006; Schneider, *et al.,* 1985; Tignor, *et al.,* 1977) approximately 300km North West of the initial isolation.

During 1986 the virus was discovered in the neighbouring country of Zimbabwe, this time it was isolated from a *Nycteris thebaica,* another small insectivorous bat species (easily identifiable due its long ears and 'slit face') (Foggin, 1988; van Eeden, *et al.* 2011). Subsequent to these aforementioned isolations there was another human case confirmed in 2006, again being linked to contact with small, most likely insectivorous bat (van Eeden, *et al.,* 2011). Once again this case was from South Africa and this time only an approximate 80 km from the original infection 36 years earlier.

Until a fatal human case in Kenya during 2007, where a tourist who came into close proximity to cave dwelling bats whilst visiting Kenya, fell ill upon return to the Netherlands (van Thiel, *et al.,* 2008), all isolates and reports of this virus species were obtained from South Africa and its'

neighbouring country Zimbabwe. This led to the assumption that the virus was geographically restricted to the southern region of Africa, either due to the climatic conditions present or by the limitation of its hosts' range. With the addition of this new case, the DUVV species has had the addition of a Kenyan lineage, suggesting that this group of viruses is not as geographically restricted as previously thought (Paweska, *et al.,* 2006; van Eeden, *et al.,* 2011; van Thiel, *et al.,* 2009).

The genetic divergence of the Duvenhage virus shows two very clear and distinct groupings. Firstly the genetic variation within the species shows that the Kenyan isolate is distinctly different from the other southern African isolates, suggesting the presence of at least two lineages of the virus, which at this point can be attributed to geospatial separation within the continent. The second factor that becomes apparent from the genetic comparison is clearly shown by the phylogenetic distances of the nucleoprotein gene. These differences allow for DUVV isolates (Paweska, *et al.,*2006, van Eeden, *et al.,* 2011) to group together, separated from the other African associated lyssaviruses and show a closer association to RABV and the other phylogroup I viruses. This divergence from the African associated viruses is supported with strong bootstrap values in excess of 85 % leaving DUVV as a separate and divergent species most closely grouping with the EBLV1, and the recently described Irkut virus (IRKV). Despite these recent isolations and the severe threat this virus pose, the limited number of cases and lack of surveillance leaves much still unknown about DUVV, including its natural reservoir and its natural geographical range (Kuzmin, *et al.* 2010; Nel, 2005).

With the inclusion of the results of this study, DUVV has only been isolated six times in the last fifty years and to date there has only been one study demonstrating any significant serological results. This study was performed on a population of *Nycteris thebaica* in the kingdom of Swaziland, which borders South Africa. The study demonstrated that as much as 30 % of the sampled individuals were in possession of neutralising antibodies against DUVV, which is the first indication of this virus circulating within this bat species in Swaziland (Markotter, Manadjem & Nel, in press).

1.2.5.3. Mokola virus

MOKV was first isolated from shrews (*Crocidura spp.*) in the Mokola forest in Nigeria in the late 1960's (Kemp *et al*., 1972; Shope *et al*., 1970). Since then MOKV has been isolated from shrews (Kemp *et al*., 1972), domestic cats (Foggin, 1983; Meredith et al., 1996; Sabeta *et al*., 2007), dogs (Foggin, 1983; Sabeta *et al*., 2007) and a rodent (Saluzzo *et al*., 1984). MOKV has also been reported to have caused two human infections (Familusi *et al*., 1972) however these cases cannot be corroborated. Thus far, all isolations of MOKV have been made on the African continent, and interestingly MOKV is the only lyssavirus other than Ikoma that has to date not been isolated from bats (Bourhy *et al*., 1993). The reservoir for this lyssavirus is not known and the epidemiology is poorly understood.

The initial distribution of cases and isolations appeared to be maintained within the equatorial regions of the continent with the majority of early isolations coming from Nigeria, but as time passed a number of cases were detected in South Africa (Sabeta, *et al.,* 2007; Swanepoel, 2004). The

distribution appears to be far more extensive with a continental dispersal pattern, suggesting proper surveillance as being the key to detecting this virus. In the late 1960s when the virus had first been isolated and active surveillance was undertaken to locate a plausible reservoir host, a single *Eidolon helvum* was found in possession of neutralising antibodies against the virus. This individual was part of 34 bats tested in a wider search for possible host animals (Kemp, *et al.,* 1972). This single case of neutralisation could have been due to inaccuracies in the testing available at the time, or due to low level background neutralisation. Although as yet there have been no studies demonstrating the host species of this virus, the lack of detection of both the virus itself and neutralising antibodies within African bat species suggests that they are not involved. However the cases of cats in South Africa succumbing to the virus, implicates a small mammal or insect as the reservoir due to the likelihood of this type of animal coming into contact with or falling prey to the felines and thus facilitating the spill overs.

1.2.5.4. Rabies virus

Rabies has been known for centuries with records dating back as early as 500 B.C. with its effects in canines being described (Steele and Fernandez, 1991). Since the identification of the ability of this disease to be transmitted from infected animals, continuous attempts to eliminate the disease have been undertaken with limited success. Most developed countries have a level of control over the disease with extensive vaccination campaigns being set in place. In contrast much of the developing world struggles with on-going cycles of the canid variant. South Africa specifically suffers with both the canid variant, affecting domestic dogs and free roaming canines, and the mongoose variant found in members of the *herpestidae* family of wild life. These biotypes circulate throughout domestic and wild carnivores playing the major role in the sylvatic rabies cycle of South Africa. The extensive distribution of these variants coupled with the social economic burdens of vaccination campaigns and the ability of free roaming animals to transport the virus not only within the country but also across borders produces a daunting situation in the fight to control this disease.

The mongoose variant is believed to have been present in the area since 1800 whilst the canid variant has a far more recent introduction dating from the 1950's from Europe (King, Meredith, & Thomson, 1993; Swanepoel, *et al.,* 1993; van Zyl, *et al.,* 2010). These variants circulate freely in members of the *Canidae* family, who maintain the spread of the virus and are the cause for many spill-over infections into non-canid species, generally leading to dead end infections with no further transmission. RABV itself has as yet been undetected in African bat populations, although in 1990, Aghomo, *et al.,*

described the detection of RABV antibodies in *Eidolon helvum* in western Nigeria. This is in contrast to American bat species which harbour their own variants of RABV with cross-species transmissions occurring between closer related bat species relatively frequently (Streicker, *et al.* 2010; Yan, *et al.,* 2001). Similarly American bat populations have been documented to have antibody levels in up to 80 % of the colony, whilst typically large colonial species display a sero prevalence of between 20 and 40 % and species that form smaller groups present less than 10 % sero positivity (Hughes, *et al.,* 2005; Turmelle, *et al.,* 2010).

1.2.5.5. Shimoni bat virus

In 2009, a bat tested positive for the presence of a lyssavirus in Kenya. During that study over 600 individuals were sampled, representing no less than 22 species from 10 distinct and geographically separate sites. The brain from a single adult female Commerson's leaf-nosed bat (previously *Hipposideros commersoni* now *H. vittatus*) found dead in a cave near the village of Shimoni reacted positively to the direct fluorescent antibody (DFA) test (Kuzmin, *et al.*, 2010). Sequence comparison to the other members of the *Lyssavirus* genus determined that it was a new virus belonging with LBV and MOKV in the former phylogroup II. This new virus was named after the area of its isolation *Shimoni bat virus* (SHIBV). Subsequently, neutralising antibodies against SHIBV were found in both *H. vittatus* and *R. aegyptiacus* with some level of cross neutralisation to LBV showing that *H. vittatus* is the most likely reservoir with possible spill-over to the roost sharing *R. aegyptiacus* (Kuzmin, *et al.,* 2011). With surveillance currently having been limited to Kenya, the

picture of distribution and host species may change as the true extent of the virus is understood.

1.2.5.6. West Caucasian bat virus

WCBV was originally isolated from the brain of an apparently healthy male *Miniopterus schreibersi* in the western Caucasus Mountains, Russia, during 2003 (Kuzmin, *et al.,* 2005). This new addition to the *Lyssavirus* genus was shown to be linked closest to LBV and MOKV, but with bootstrap values of less than 68 % did not fall into the former phylogroup II (Botvinkin, *et al.,* 2003). WCBV was therefore seen as the most divergent member of the lyssaviruses and the lone member of its own proposed phylogroup III until the discovery of the putative Ikoma lyssavirus (Marston, *et al.,* 2012c) (as seen bellow in section 1.2.5.7.). This divergence from the other lyssaviruses and the lack of evidence of this species on the African continent prior to the discovery of neutralising antibodies in Kenya prompted argument over the continental origin of the progenitor species of this virus and indeed all lyssaviruses. With the level of divergence present in this non-African associated virus placing serious doubt on the origins being African. With convention explaining the migration of ancestral bats from Africa to the other continents and taking the progenitor lyssavirus with them as they dispersed, it was expected that a viral species this divergent from the others would be of an African association. This debate has lessened with the discovery of WCBV neutralising antibodies in Kenya and the new isolation of IKOV (Kuzmin, *et al.,* 2008a; Marston, *et al.,* 2012a)

During sampling in 2006 and 2007 antibodies capable of neutralising WCBV were identified from *Miniopterus spp.* in Kenya (Kuzmin, *et al.,* 2008a). This discovery has reaffirmed the theory of Africa being the cradle of lyssaviruses. Despite the presence of neutralising antibodies there are no viral isolations on the African continent as yet. There is also no active surveillance in place and despite *Miniopterus spp.* being implicated the reservoir species has not been isolated.

1.2.5.7. Ikoma lyssavirus

The most recently proposed member of the lyssavirus group, Ikoma lyssavirus is only known from a single sample, isolated from an African civet in north-west Tanzania. The new variant appears to be the most divergent of all the species described thus far, with a maximum sequence identity in the nucleoprotein gene of only 68.8 % with WCBV. This putative species was found in a rabid African civet (*Civettictis civetta*) near the Serengeti National Park, Tanzania, in the Serengeti and Ngorogoro district (ICTV 2011; Marston, *et al.,* 2012a; Marston, *et al.,* 2012c). Although this isolation was from a civet, it is believed more likely to be as a result of a spill-over event from the reservoir host. Although civets in this region have not been screened, a previous survey in southern Africa did not demonstrate any civet specific lyssaviruses (Sabeta, *et al.,* 2008). The hypothesis of this case being as a result of a spill over event is further strengthened by the fact that these nocturnal hunters inhabit areas near and frequent caves where they may come into contact with roosting bats, suggesting the origins of this virus as a bat-associated lyssavirus.

1.2.6. Chiropterans associated with lyssaviruses in southern Africa

Surveillance for the rabies-related lyssaviruses is limited with the prevalence throughout southern Africa unknown. The old world frugivorous bats, of the family *Pteropodidae* are generally considered and accepted as the most likely reservoir species of Lagos bat virus, whereas insectivorous bats such as *Miniopterus spp. and Nycteris thebaica* are the suggested reservoirs of Duvenhage virus. Likewise the widespread *Hipposideros vittatus* is the reservoir of the recently isolated Shimoni bat virus. All of these bat species are present throughout the southern African region and within South Africa, making it plausible for the associated viruses to be in the region as well.

The African bat associated lyssaviruses have been encountered throughout the entirety of the continent, wherever there has been comprehensive surveillance. Most notably; several new Lagos bat virus isolates have been isolated from bats (*Ep. wahlbergi)* in the Kwa-Zulu Natal region of South Africa, in the past decade (Markotter, *et al.,* 2008a). This was after a period of 13 years prior to that with no reported encounters with this virus. In addition the virus was isolated, for the first time, from non-volant wildlife (namely; a mongoose) (Markotter, *et al.,* 2006a) and has been associated with failure of the rabies vaccines in a dog (Markotter, *et al.,* 2006a; Nel, 2005; Ullas, *et al.,* 2012). Several isolations of MOKV have also been made in South Africa from cats in recent years, also after a long period of apparent absence (25 years) (Sabeta, *et al.,* 2007). Duvenhage virus has

recently been isolated in South Africa and Kenya (Koraka, *et al.,* 2012; van Eeden, Markotter, & Nel, 2011; van Thiel, *et al.,* 2008; van Thiel *et al.,* 2009). Both these recent cases have been in humans, after bat exposures, but the specific bat species involved in transmission is as yet still unknown.

Despite the presence of these bat associated cases, it is not to be said that these viral species have been limited to bat populations, as many other species have been identified in spill-over infections into terrestrial mammals including humans. However, the fact that all the terrestrial mammal infections appear to be as a result of individual species jumps from the bat species reservoir, lends strong evidence to the existence of an ancestral condition or progenitor virus in the distant past which evolved and diversified with the bat populations as they dispersed and colonised the continents (Delmas, *et al.,* 2008).

1.3. Surveillance strategies for lyssaviruses in bats

With bats being typically reclusive and rarely coming into direct contact with humans, monitoring their behaviour and the state of their populations has historically been neglected, leaving many questions about their habits still unanswered. The full extent of the pathogenesis of viruses in their bat hosts is not completely understood and despite the recent flourish of research, the bat species that are affected by these viruses are not always known. In order to take the first step in addressing the fundamental questions, focused surveillance of bat populations is required. This surveillance can address two major factors by two different approaches. The first of these is to determine which viruses are present within a specific bat specie, this is achieved by

searching for viral genomic RNA and where possible isolating the virus. This allows for definitive proof as to which viral species are present in that specific population and conversely this shows which virus species the bat population is susceptible to, as only an active viral infection in the bat will yield viral RNA. The second approach, aims to determine the extent to which the bat population has been exposed to and come into contact with the virus of interest. This is achieved by screening for antibodies capable of neutralising specific viruses, thus indicating that the animal had been exposed to that virus or a cross neutralising relative before. This approach has the added benefit of demonstrating (if performed properly), which cross neutralising virus groups are most likely circulating within that bat population, by determining which viruses can be neutralised and thus which viruses the bats have been exposed to. From the combination of these two results, the extent to which these bat populations are exposed to these cross neutralising virus groups and the specific viruses present within distinct bat populations can start to be assessed.

1.3.1. Active infection

To ascertain if a suspected animal is suffering from a lyssavirus infection it is necessary to either identify the presence of the entire virus or of the viral genomic RNA. This detection of viral infection can be facilitated by either sampling the brain material directly, requiring the death of the sampled individual, or by the far less reliable salivary swab which may be able to detect shed virus. The direct approach is by far the more reliable and effective with the excretion of virus via the salivary glands differing in viral titre and only occurring sporadically. This means that with only the use of salivary swabs the virus may be missed completely. Both of these strategies can be used for the detection and possible isolation of the virus itself, however brain samples are far more reliable for detection and due to the higher titres reached allow for more efficient and successful viral isolation.

1.3.1.1. Fluorescent Antibody Test

The fluorescent antibody test (FAT) is the gold standard for the diagnostic testing of lyssavirus infections (Dean *et al.,* 1996) with the test being able to be performed on a multitude of sample types, with the most accurate and reliable being brain tissue. A smear or an impression of the tissue is made and then treated with anti-lyssavirus conjugate labelled with a fluorescein isothyocyanate (FITC) marker. This treatment facilitates the fluorescence of the bound conjugate under fluorescent magnification (Wiktor, *et al.,* 1980) whilst non-bound conjugate is removed during washing steps. This test produces varying degrees of positive responses depending upon the specificity of the monoclonal or polyclonal antibodies in use and the lyssavirus being detected and the state of the sample material (Bourhy, *et al.,* 1993). If monoclonal antibodies are being used, their specificity may be used to type the virus antigenically. Monoclonal antibodies will only bind to viruses presenting the required attachment sites, so with a panel of antibodies the members of the genus can be distinguished. In South Africa a polyclonal antibody (Onderstepoort Veterinary Institute, Rabies Unit, South Africa) is used to perform the FAT. This FAT is capable of detecting all of the lyssavirus species known to be present within the country.

1.3.1.2. Viral nucleic acid detection

During the course of a viral infection, the virus must use the host cell to produce the components necessary to construct additional viral particles and facilitate the spread of the virus. Arguably the most essential portion of the new viral particles is genomic information, which holds the ‘blueprint’ for all

viral function and consists of RNA in the case of lyssaviruses. The fundamental premise upon which molecular viral detection is based, is amplifying the typically low levels of genomic information present within a sample to detectable and usable amounts by means of an enzymatically based polymerase chain reaction. There are at present several reincarnations of the PCR reaction, all of which revolve around the process of converting RNA to DNA if necessary and then replicating the DNA to higher amounts.

The fundamental difference between the most commonly used conventional PCR and the “new” real-time PCR is the manner in which the intended amplicon, showing a positive result, is detected. The real-time detection relies upon the measurable accumulation of the amplicon product, cycle by cycle, and the associated fluorescents from oligoprobes, whilst the conventional detection is performed only after cycling is complete by means of UV-irradiated agarose gel electrophoresis and a dye capable of intercalating with DNA (Mackay, *et al.,* 2002). For the purposes of viral detection the real-time approach not only provides a quicker result but also provides a more accurate analysis of the sample, with detection of very low viral loads being possible (Coertse, *et al.,* 2010).

A PCR designed for the purpose of detecting a number of different species within a specific group will ideally rely on its primers binding to a specific region of the genome that is conserved between all the members but unique to the group. For this reason the N-gene is the most logical choice for the development of a protocol for the detection of African associated lyssaviruses despite the limitations for the differentiation between isolates due to the inherent conservation of this gene region. The primers 541lys and 550B

and probe lyssaprobe620 used by Coertse, *et al.,* 2010 offer the best results for the detection of the African associated lyssaviruses (Coertse, *et al.,* 2010; Markotter, *et al.,* 2006). In contrast to the other real-time detection methods available at present, the Coertse, *et al.,* 2010 system is able to detect all of the variants of lyssavirus currently known within South Africa, where as the other probe based systems are primarily designed to detect the variants in the area of the world they were developed or at best, seven of the species (Vazquez-Moron, *et al.,* 2006; Nadin-Davis, *et al.,* 2009) whilst the syber green systems have limitations in the level of detection (Hayman, *et al.,* 2011). The real-time technique also allows for detection of viruses at theoretically single copy number rather than the higher values required by the hemi-nested PCR systems (Heaton, *et al.,* 1997). These systems of nucleic acid detection, all demonstrate the presence of actively replicating virus within the sample being investigated.

1.3.1.3. Virus isolation

Detection of active infection may also be achieved by the process of virus isolation. This technique revolves around the process of transferring infectious material from the original host to a susceptible laboratory animal or cell culture. After appropriate incubation periods the methods described in section 1.3.1.1. and 1.3.1.2. can then be used to characterise the virus. However, this method does pose problems with the susceptibilities of either the animal model or the cell culture to the virus.

1.3.2. Exposure to lyssaviruses

The aim of lyssavirus serological surveys is to determine the exposure of a group of animals towards a specific virus by detecting whether there are virus-neutralising antibodies present within that population. Neutralising antibodies may occasionally be produced in the later stages of the disease progression by animals that succumb to rabies and are also the intended result of vaccinations, but are typically expected within reservoir hosts that do not allow progression of the virus. The presence of these antibodies in the blood sera of apparently healthy, wild animals is a fair indicator that the animal has been exposed to the virus being screened yet the virus has not progressed to its typically lethal state. It is important to note; the presence of antibodies in apparently healthy animals does not mean presence of the virus, it indicates only that the animal has been exposed to the virus and in most cases, if the animal is still alive, overcome the challenge of that virus. In contrast to viral detection, this serological detection process is far less invasive and normally leaves the sampled individual alive, this system is used for demonstrating that the virus is or has been present within that population rather than aiming to isolate the virus.

Viral neutralisation tests are the most sensitive and arguably the most reliable for the detection of viral-antibodies. The fluorescent antibody virus neutralisation (FAVN) assay and the rapid fluorescent inhibition test (RFFIT) are the most recognised neutralisation assays, with comparable results, as demonstrated by Briggs, *et al.,* 1998. For the purpose of this project the RFFIT is preferable due to its ability to be miniaturised as Dzikwi, *et al.,* showed in 2010. This modification allows for the use of smaller volumes of sera to be used, typically for the RFFIT a volume of up to 100 µl of sera is used whilst volumes of no more than 7.5 µl of sera are sufficient with the miniaturisation. This substantial reduction in volumes required will be of great benefit for this study as the blood available from the bats samples will be very limited from the majority of individuals due to no more than three percent volume blood per body mass being removed.

B. Chiroptera

1.4. Evolutionary history and distribution

As is found with the distribution of the lyssaviruses, bats are found to have a near global distribution inhabiting every continent, with the exception of Antarctica (Monadjem, *et al.,* 2010; Simmons, *et al.,* 2008). Chiroptera perform essential ecological functions throughout the plethora of ecosystems they inhabit, acting as both predators of insects and pollinators of flowers. This diverse order of mammals is not limited to just these food sources and unlike other mammalian species many of the species may happily coexist in large numbers of not only the same species but also with multiple species within the same system. The chiropteran order comprising of some 1100 recognised species constitutes at least 20 % of all known mammalian species described thus far. This diverse order of mammals retains three major features separating it from other mammals; 1) the ability of true powered flight, 2) echolocation and 3) comparative longevity in comparison to body size (Simmons, 2005; Simmons, *et al.,* 2008; Turmelle & Olival, 2010).

Most likely due to the animals' small size and the implicitly delicate nature of the bone structure there are extensive gaps in the fossil record for chiropterans, with reported coverage of only approximately two percent of the megabats and between 14 and 44 % of the microbat history (Simmons, 2005). Due to the continuing conflicts between molecular and morphological data, the higher level phylogeny of bats has been continuously reshuffled and reassigned through the years. (Simmons, 2005; Teeling *et al.*, 2005; van den Bussche and Hoofer, 2004). The order C*hiroptera* was historically divided into two suborders; 1) the *Megachiroptera,* containing *Pteropodidae,* the old world fruit bats with

limited echolocation capabilities, and 2) *Microchiroptera* consisting of typically small insectivorous bats possessing a far more developed echolocation system. This traditional grouping was forced into review after phylogenetic findings demonstrated that the *Pteropodidae* family of 'megabats' and the previously proposed *Rhinolophoidea* superfamily of the former *Microchiroptera* were far more closely related than was previously thought. This genetic relationship was demonstrated to be far more substantial than the relatedness of the *Rhinolophoidea* to the rest of the insectivorous 'microbats' *Microchiroptera.* Thus the modern grouping of the suborders; 1) Yinpterochiroptera, containing both *Pteropodidae* and *Rhinolophoidea* and 2) Yangochiroptera made up of all other bat species was decided upon (Teeling *et al.*, 2005). To facilitate this newly designated nomenclature there was the necessity of the incorporation of multiple superfamily, sub- and infra- order designations as shown in Table 1.3 (van Cakenberghe, & Seamark, 2011).

Despite the on-going research into the diversity of chiroptera, the phylogeny is poorly understood, with traditional classes of classification being insufficient for this order. This confusion is further compounded by the lack of understanding available to explain the adaptive radiation and distribution seen within these animals. Thus for the purpose of this review and for ease of description and referencing the classification followed by the 2011 African Chiropteran Report (ISSN 1990-6471) will be used. The initial description of this rearrangement system was put forward by Hutcheon and Kirsch in 2006, and relies upon the dissemination into two suborders, namely *Pteropodiformes* and *Verpertilioniformes.*

Presently there are a total of eighteen extant chiropteran families based upon shared anatomical characterisations, echolocation frequencies and behaviour. Additionally to these, six extinct families have been proposed from the available fossil records. The phylogenetic separation of the families and species has been demonstrated to have only occurred relatively recently helping to explain some of the genetic ambiguity currently confusing classification systems (Eick *et al.*, 2005; Teeling *et al.,* 2005).

The earliest distinctly bat fossil records of the chiropteran order date from the early Eocene period of the Cenozoic era an estimated 56 million years ago (mya) (Teeling, *et al.,* 2005). Despite the age of these fossils all representatives found thus far demonstrate the specialisations needed for powered flight and echolocation, thus the true origin of bats must pre-date these fossils. There are two trends of thought about the origin of bats currently available; The first as put forward by Teeling *et al.,* (2005) based upon the concatenated sequence of seventeen nuclear genes suggests a Cenozoic era origin in the Northern Hemisphere with a most recent shared ancestor some 64 mya, with subsequent differentiation and dispersal from that point. The second proposed origin was put forward by Eick *et al.,* (2005), using the sequence of four nuclear intron marker unique to bats and dispersal-vicariance modelling. This second system proposes an African origin some 65 mya, with multiple dispersals and subsequent vicariance events occurring as the burgeoning order radiated out across the globe.

Table 1.3. Higher order classification of the African associated Chiroptera as outlined in the 2011 African Chiropteran Report (ISSN 1990-6471).

Suborder	Infra order	Super family	Family	Subfamily
Pteropodiformes	*Pteropodiformacei*	*Pteropodoidea*	*Pteropodidae* (fruit bats, previously; mega bats)	*Pteropodinae*
				Macroglossinae
				Propottininae
	Rhinolophiformacei	*Rhinolophoidea*	*Hipposideridae* (Leaf nose bats)	*Hipposiderinae*
			Rhinolopidae (Horseshoe bats)	
			Megadermatidae (False vampire bats	
		Rhinopomatoidea	*Rhinopomatidae* (Mouse tailed bats)	
Verpertilioniformes	*Noctilioniformacei*		*Noctilionidae*	
			Myzopodidae	
	Nycteriformacei	*Nycteroidea*	*Emballonuridae* (Sheath tailed bats)	
			Nycteridae (Slit-faced bats)	
	Vespertilioniformacei	*Molossoidea*	*Molossidae* (Free-tailed bats)	*Molossinae*
			Miniopteridae (Bent-winged bats)	
			Verpertilionidae (Common bats)	*Kerivoulinae*
				Myotinae
				Scotophilinae
				Vespertilioninae

Both of these proposed systems present an evolutionary scenario where there was rapid speciation and change in the population during the Eocene some 60-52 mya, most likely due to the extensive conversion of forested to woodland areas at that time thus providing a plethora of new feeding opportunities, resulting in the differing adaptations in both flight patterns and echolocation strategies (Miller-Butterworth *et al.*, 2007; Teeling *et al.*, 2005). However, neither proposed origin fully explains the distribution of the present families, with some members appearing on separate continents with no visible link existing. To emphasise this point, the case of *Noctilionidae* and *Natalidae* as raised by Eick, *et al.,* 2005 is used. The evidence of gene flow between member species of these families currently found in the Americas and Africa stopped only an approximate 30 mya, relatively recently, despite the continents having separated between 84 and 100 mya. This unanswered question has led to the development of three competing scenarios to explain the radial expansion of bat species. These scenarios are; 1) direct movement across the early Atlantic Ocean, utilising land masses, reduced sea levels and or the occurrence of vegetation bundles, 2) the dispersal through Eurasia and use of the 'Beringa' land mass to obtain access to North America and subsequent dispersal and 3) the use of small island chains present between the Americas and Africa which would have been more accessible in the past.

1.5. Bat species in southern Africa

The southern African bat populations cover at least 116 species from 18 family groups of chiroptera with members of both the large fruit eating bats *Pteropodiformes* and the smaller insectivorous bats *Vespertilioniformes*

(Monadjem, *et al.,* 2010). Of the known species, five have been moved onto the Global red list of vulnerable animals whilst a further 17 are considered near-threatened. There are however 17 other species that have insufficient data with which to be evaluated (Monadjem, *et al.,* 2010). It is commonly believed that the known species are not the full extent of the species diversity within southern Africa, and as surveillance is increased and techniques such as genetic characterisation are developed further this number of unique species will continue to increase.

1.6. Identification of bat species

1.6.1. Morphological classification

For morphological identification the first step in the process is visual assignment of the individual, this allows placement into general groups, normally at a genus level. For species lacking specific distinguishing features or sharing general morphology with closely related members, typically the measurements of the forearm and skull are used along with the dentition of the animal to place the animal into its species class according to extensive keys (Meester *et al.,* 1986; Monadjem, 1997; Taylor, 2000). After a general identification has been performed with the use of these general keys a more refined classification can be used for specific family groups, such as Bergmans (1997) for *Epomophorus spp.*, Robbins, *et al.* (1985) for clarifying *Scotophilus spp.,* and *Rhinolophus spp.* can be resolved using Csorba *et al.* (2006).

The baculum morphology of the male individuals of the *vespertilioniformes* is also a key component for obtaining clarity between similar species (Csorba *et al*., 2006; Hill and Harrison, 1987; Kearney *et al*., 2002). These morphological measurements are currently the most precise manner by which to assign an individual however, the process of measuring can yield differing results depending on the techniques employed and accuracy of the examiner. There are also areas of confusion when a measured individual falls within the guidelines of more than one species. These grey areas in the keys require that all measurements be available to make an as accurate identification as possible, thus requiring the entire carcass to be available.

This lengthy process of measuring and comparing is typically handled by dedicated staff in museums where the vouchers are kept for future reference. It is at this point where some of the more interesting speciation is noticed, when a measured individual is compared back to the holo-type or original voucher for that species and found to be well outside the norm. It is also through this retention of original material that it is possible to go back and observe how species variance and presence have shifted in some areas over time.

1.6.2. Echolocation

The majority of bat species are able to use echolocation for navigation and foraging. This facilitates a view of their nocturnal world by emitting an ultrasonic call via the nose or mouth, typically between 20 and 60 kHz (Acharya & Fenton, 1992) and listening for the returning echo. As described by Eick *et al.,* in 2005, these echolocation calls are divided into two independently evolved systems; the high duty-cycle where a long high pitched frequency is emitted, which may overlap with the returning echo. The low duty-cycle calls are typically at a lower frequency and with longer delays between each call. These systems reflect the flight pattern used by the bat in its foraging environment, with the continuous high duty-cycling calls typically being used in very confined and cluttered environments where slow flight is essential.

Echolocation calls are unique in frequency and duration for specific species, thus facilitating the identification of bats by their calls (Monadjem, *et al.,* 2010). This system has been used to great effect in some regions of the world (Vaughan, Jones, & Harris, 1997) but there are the possibilities for complication with overlapping frequencies within species groups and with individual bats producing different calls during normal flight which may lead to incorrect identification.

This process of monitoring and observing bats via their echolocation is facilitated with the use of so termed ‘bat-detectors’ which record the ultra sound signal produced from the bat and through a process of time expansion and amplification provide a new sound audible to humans. These detectors regardless of their level of sophistication provide information as to the frequency of the incoming signal allowing the generalised determination of the species producing the call.

1.6.3. DNA barcoding

In 1994 Folmer and colleagues described a 640 nucleotide region at the 5’ end of the mitochondrial cytochrome oxidase subunit I (COI) gene, that was proposed as a tool for the unique identification of specimen’s to a species level. The importance of this gene product for aerobic metabolism has facilitated its presence in practically all living animals and has been largely removed from selection pressure. This existence within the mitochondrial genome has propelled it to become the most popular region for the species specific identification of specimens and the identification of new species in conjunction with the ‘barcoding of life database’ (BOLD) initiative (Hebert, *et al.,* 2003; Stoeckle, 2003). The Barcoding of life initiative is formed by a consortium of international organisations including global herbariums and natural history museums, which planned a process for the rapid and inexpensive identification of all of the world’s species. This gene based identification process is achieved by using short DNA fragments, divergent enough from one another to show species differences, yet still conserved enough to be present in all forms of life, thus facilitating its universality.

However at this point in time there are a number of genes facilitating the barcoding duties for the different forms of life, with the COI region gaining favour for the barcoding of animal life.

Since the development of this molecular tool it has been used with many animal groups, but with bats in particular it has provided a clearer understanding of the small mammal diversity present in the tropical areas of South East Asia where deforestation and human encroachment threaten to remove species before their actual discovery (Francis, *et al.,* 2010). The first real attempt to quantifying the species diversity present within the neotropic bat populations undertaken by Clare, *et al.,* 2011*,* provided the preliminary evidence of population separation and differentiation between inland and coastal bat populations. This work has raised the interest in using the technique in similar programs and has demonstrated that the vast diversity already seen in the bat populations is only the starting point in understanding their complexity. Additionally the production of DNA barcodes of all Canadian bat species, which has aided in the on-going rabies surveillance by allowing species identification without need of a carcass, has allowed for more effective planning to be implemented with species being targeted rather than a generalised response (Nadin-Davis, *et al.,* 2012). This has been facilitated by the fact that affected individuals are of a DNA barcoding confirmed species with its known roosting preference and general geographical distribution taken into account, allowing for the risk to be assessed.

In contrast to these success stories, there have been limitations and problems experienced with this system as described by Nesi and co-workers (2011) during their work on African fruit bats. Although they agree on the

inherent advantages of DNA barcoding their work shows that there are limitations in the resolution available from these sequences. Their study illustrates that COI sequences of *E. gambianus* and *Micropteropus pusillus* do not discriminate between the species. From these results and the fact that species level resolution is obtained using the β-fibrinogen, it has been suggested to be used as an alternative DNA barcoding gene for mammals.

The clarity theoretically provided by DNA barcoding, removes the need of the entire carcass being taken and will remove any measuring bias that may occur. This system allows for accurate species detection from only a small sample of tissue regardless of the origin, although tissues with a higher yield of DNA will provide better results. Similarly, properly preserved historical or partially decomposed samples that are in poor condition can also be accurately identified, if sufficient DNA can be isolated, allowing for the archival samples from around the globe to be processed. If the barcode of the holo-types of species becomes available the question of speciation and incorrect identification can be addressed more acutely. This would also negate the need of transporting new vouchers to the institutes housing the holo-type for comparison. Since its introduction COI has already been used in different disciplines e.g. the identification of cryptic vertebrate species in Australia, determining the insect diet of bats, identifying species in Guyana and investigating species diversity of African birds and fish (Clare, *et al.,* 2006; Fenton, 2013; Goerlitz, *et al.,* 2010; Oliver, *et al.,* 2009; Ward, 2009). More specifically in regards to the bats of Africa, Nesi *et al*., , 2011, has used the system for identifying fruit bat species, whilst Monadjem, *et al.,* 2013, have

determined the diversity of *pipistrelloid* bats showing the capability of this technique.

This ability to identify a species from small pieces of tissue negates the need to take vouchers of individuals for morphological identification during the course of surveillance programs, allowing investigation of even endangered species to be performed. The system of DNA identification also lends itself to situations where the identification of the host animal is outside of the primary expertise, allowing accurate and reliable identifications of the animal host.

1.7. Aim of study

1.7.1. Perform serological lyssavirus surveillance of South African bats by testing serum samples for the presence of neutralising antibodies against Duvenhage virus, Lagos bat virus and rabies virus.

1.7.2. Screening of all available bat brain tissue samples for the presence of lyssavirus genomic RNA, generating DNA sequences of positive samples and phylogenetic analysis.

1.7.3. To generate COI DNA barcode sequences of selected morphologically identified South African bat species and creation of a preliminary reference COI database for South African bats.

Chapter 2

Lyssavirus nucleic acid detection and sero-surveillance in South African bat species

2.1. Introduction

The southern African region is known to host a number of the lyssaviruses, including at least two variants of RABV as well as MOKV, LBV and DUVV. To address the prevalence of lyssaviruses within the South African bat population two major strategies were employed. The first of these was serological detection of antibodies capable of neutralising DUVV, LBV or RABV. This provides an indication of the presence of lyssaviruses within the different populations of South African bats. MOKV was omitted from this serological screening of the South African bat populations due to its cross neutralisation potential with LBV that could cause confusion in the results coupled with the lack of substantial evidence for this virus being found within any bat populations. Conversely RABV was included to allow for partial normalisation of samples, as African bats are not anticipated to have exposures to this species of lyssavirus, the presence of neutralising antibodies against RABV would most likely indicate a level of background neutralisation rather than true exposure.

For serological surveillance there are several techniques available, yet for the purpose of this study a modification of the RFFIT (the mini RFFIT) was used. The RFFIT relies upon the addition of serum from the test animal being added to actively growing cell culture medium and challenge virus dilution. If there are antibodies present within the serum sample capable of neutralising

the challenge virus, this will be observed in the staining process as an absence of infected cells (Briggs et al., 1998). Conversely, if there are no neutralising antibodies present within the sample, the cell culture will display signs of infection by the challenge virus. In addition to this presence or absence testing, by producing an increasing dilution series of the test serum it is possible to quantify or determine a neutralising antibody titre. Due to the limited volumes of serum obtained from individual bats, the miniaturised RFFIT (Dzikwi et al., 2010) was considered the preferred method of screening.

Secondly the presence of viral RNA was tested for in the brain material of potential hosts. Previously conventional reverse transcription polymerase chain reaction (RT-PCR was used (Vazquez-Moron, *et al,* 2006), but recently the rapid and more sensitive real-time RT-PCR has been developed (Coertse, *et al,* 2010). The amplification process relies upon the presence of viral RNA in the sample. This RNA has to be of such quality (non-degraded) that amplification of the entire region of choice is allowed. For the purpose of this study, a published real-time RT-PCR method was followed, in which a portion of the nucleoprotein gene is amplified (Coertse, *et al.,* in 2010). This method was designed to and is known to specifically detect all known lyssaviruses present within South Africa at even low copy numbers thus providing the best opportunity for accurate viral detection.

The sporadic shedding of viral particles in saliva and dispersal of the virus to other organs during the progression of the disease leaves the brain material as the most reliable and likely to yield viral RNA. The detection of one lyssavirus from the 413 samples tested provides direct evidence of viral

infection. In contrast to this 0.24 % the detection of neutralising antibodies in 33 % and 13 % of the individuals screened for LBV and DUVV respectively indicates the level of exposure to these viruses. Both of these strategies play important roles in the surveillance program, providing further evidence of *Nycteris thebaicas'* implication in the circulation of DUVV, with the latest isolation of this specie, and strengthening the knowledge of LBV circulation in *Epomophorus wahlbergi* whilst the serological results demonstrate that exposure to lyssaviruses is far more extensive than previously anticipated.

2.2. Materials and Methods:

2.2.1. Sample collection

From the samples collected, either by mist net or harptrap (Kunz, Hodgkison, & Weise, 2009) and the 62 samples submitted by the public from areas throughout South Africa as outlined in Figure 2.1. a total of 413 brain samples and the 350 serum samples were collected. Whilst all brain samples came from voucher specimens, not all animals were bled for serological screening and not all samples that were bled were taken as vouchers, leaving only a partial overlap in the samples.

All samples were collected as per the relevant permits from the appropriate authorities; Samples collected in the areas of Taung and Madikwe in the North West Province of South Africa under the permit number 000039 NW-07. The collection of samples from the Rocktail Bay area of Kwa-Zulu Natal, South Africa was performed as per the guidelines set out in the permit OP 500/2010.

The Pafuri samples were obtained under the agreement between Dr. Teresa Kearney, SANParks and Makuleke Communal Property Association for research on 'Inventory of bat species occurring at Pafuri (Makuleke Contractual Park), with a comparison of morphological and molecular identifications, and screening of voucher specimens for viruses'. Voucher specimens were removed from the park under permit number RB/2010/04.

Bats sampled in the Limpopo province and taken as museum voucher specimens were done under the permit obtained from the Department of Economic Development, Environment & Tourism (LEDET) of the Limpopo provincial government, CPB6 – 003767. A total of 62 samples were submitted to the University of Pretoria for rabies testing after the bats had been found dead or had been involved with human contact, five of these samples were also used for DNA barcoding and three samples used for serum screening.

1. Durban, Kwa-Zulu Natal 2. Amanzimtoti, Kwa-Zulu Natal 3. Allerton, Kwa-Zulu Natal 4. Louis Trichard, Limpopo 5. Melmouth, Kwa-Zulu Natal 6. Irene cave, Gauteng 7. van der Kloof, Northern Cape. 8. Kwasmane nature reserve, North West 9. Rooiberg, Limpopo 10. Johannesburg, Gauteng 11. Madikwe game reserve, North West 12. Matlapitsi cave, Limpopo 13. Pafuri camp, Kruger National Park, Limpopo 14. Pretoria, Gauteng 15. Rock tail bay: St. Lucia, Kwa-Zulu Natal 16. Taung, North West

Figure 2.1. Major sampling localities of bat species throughout South Africa.

2.2.2. Species identification

After initial visual identification, the more in depth morphological identification of voucher specimens was performed by Dr. Teresa Kearney of The Ditsong National Museum of Natural History, (formerly Transvaal Museum), Pretoria, South Africa, using Meester *et al.* (1986), Taylor (2000), and Monadjem (1997). Additionally, more refined classification was used for specific family groups; Bergmans (1997) was used for *Epomophorus spp.*, Robbins *et al.* (1985) for *Scotophilus spp.,* and *Rhinolophus spp.* were resolved using Csorba *et al.* (2006). The baculum morphology of the male *vespertilioniformes* individuals was also analysed according to the guidelines in Hill and Harrison (1987), Kearney *et al*. (2002), and Csorba *et al*. (2006).

Specimens from the families of Rhinolophidae and Vespertilionidae were further analysed based on measurements of both the cranium and dentition. These measurements were compared to those identified in Kearney and Taylor (2011) for the Vespertilionidae, whilst an unpublished dataset of measurements in Csorba *et al*. (2006) was used for Rhinolophidae. This allowed association within large datasets of ca. 602 and ca. 320 individuals respectively. These datasets include many type specimens representing species from across Africa, albeit with a partial bias towards the individuals found within the southern African region.

2.2.3. Serological surveillance

2.2.3.1. Collection of blood samples

Samples of whole blood were collected from 350 individual bats (Appendix S1) throughout South Africa (Figure 2.1). These samples were collected by means of either hypodermic needle and syringe or by means of venial puncture and glass capillary (Voigt & Cruz-Neto, 2009). The hypodermic needle and syringe system was used to collect blood by means of cardiac puncture (ventricle of the heart), drawing up to three percent volume per body mass of the individual. The anaesthesia of individuals was achieved by the intra muscular inoculation of a ketamine and xylazine solution with 35 mg/kg body mass and five mg/kg body mass respectively. Anesthetised individuals were restrained by a bite-proof, leather gloved hand during sampling, whilst individuals to be taken as vouchers received a euthanizing over-dose of the solution and were bled dry. Blood collected by 80 µl glass capillary (Superior Marienfeld Laboratory Glassware, Germany) were done so by puncture of the propatagial vein in the antebrachium of the wing or the caudal vein in the uropatagium (tail membrane) (Voigt & Cruz-Neto, 2009), glass capillaries were placed against the pooling blood and allowed to collect up to 50 µl this was then forced out of the capillary by means of 100 µl pipette into collection tubes. In both cases the whole blood was immediately transferred to a MiniCollect® 0.8 ml serum separator tube (Greiner Bio-One) and then stored at four °C. The blood containing MiniCollects were centrifuged at 4,300 x g for 10 minutes, allowing separation of the serum from the whole blood. The serum was transferred by means of pipette to sterile 1.5 ml eppendorf tubes and incubated at 56 °C for 30 minutes to facilitate the heat

inactivation of the complement proteins and to reduce the threat of any residual pathogens. After the completion of heat inactivation the serum was stored at -20 °C until the assays could be performed, whilst the remains of the whole blood were stored at -80 °C.

2.2.3.2. Rapid Fluorescent Focus Inhibition Test

The miniaturised Rapid Fluorescent Focus Inhibition Test (RFFIT) as described by Kuzmin *et al.* (2008a), based on the original system by Smith (1996), was used with slight modifications, to determine whether the acquired bat samples collected throughout South Africa possessed antibodies capable of neutralising lyssavirus particles. The miniaturised test was prepared on eight well, six mm Teflon coated Cel-Line slides (Thermo Fisher Scientific). An initial volume of seven µl DMEM-F12 (1:1) with L-Glutamine and 15 mM Hepes (Lonza) supplemented with 10 % foetal bovine serum (Lonza) and one percent antibiotic-antimycotic cocktail (10000 units penicillin, 10000 µg streptomycin and 25 µg amphotericin per ml, utilising penicillin G, streptomycin sulphate and amphotericin B as Fungizone®, Invitrogen Life Technologies, Gibco) was added to each six mm well. A volume of 1.75 µl of the heat inactivated sample sera was added to the first well of each slide and mixed by pipetting. Subsequently a volume of 1.75 µl was serially transferred from the first well to the next in sequence and then repeated in order on each slide, producing a fivefold dilution series of the sera from each sample. To each of these wells now containing the fivefold dilution series a volume of seven µl of the specific challenge virus was added. For each of the three challenge viruses, virus standard-11 (CVS-11), Lagos bat virus 1999 (LBV

Africa) and Duvenhage 2006 (DUVV'06) the Spearman-Karber method was followed (Karber, 1931; Spearman, 1908) to produce stock virus at a concentration of approximately $10^{4.5}$ $TCID_{50}$/ml. Control slides were prepared by means of a back titration of each challenge virus by adding seven µl DMEM-F12 (Lonza) to the wells of the slide, adding seven µl of a 10-fold serial diluted challenge virus (effectively forming a 1:20 dilution) and leaving one well per slide uninfected as a control for the cell culture. The slides containing the mixture of challenge virus and serum and the control slides were then incubated for 90 minutes at 37 °C and 0.5 % CO_2 in a prepared humidity chamber. After which 14 µl of freshly re-suspended cells was added to each well, equating to approximately 5 x 10^5 MNA cells per ml, and incubated again at 37 °C with 0.5 % CO_2 for 22 hours.

After the completed incubation, the remaining cell culture supernatant of the slides was decanted and the slides were dip rinsed in phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3) (Lonza) and transferred to ice cold acetone (Merck) and maintained at four °C for 30 minutes. After acetone fixation, the slides were air dried prior to the addition of seven µl of conjugated polyclonal rabies immunoglobulin (Onderstepoort Veterinary Institute) labelled with fluorescein isothyocyanate (FITC) at a dilution of 1:300 with the addition of Evans Blue (0.5 % in PBS), was added to each well and incubated at 37 °C for 30 minutes. After this incubation the conjugate was removed from the slides by means of two times PBS [1 x] (Lonza) rinses for 10 minutes each. The slides were then allowed to air dry again for 10 minutes before being read. Slides were read on fluorescent microscope (Carl Zeiss Axiovert 25 CFL

with filter set blue excitation 450 – 490 nm) under 160 to 200 x magnification. For each well of the slide 20 focus fields were observed for the presence of lyssavirus induced fluorescence. Each sample was tested in triplicate for each of the challenge viruses, after which statistical analysis of the results was performed using http://www.vassarstats.net/prop1.html to assist in calculations of the 95 % confidence intervals with a continuity correction.

The choice of challenge virus in the RFFIT was of specific concern, with different levels of cross neutralisation between the member species of the phylogroups (Badrane, *et al.,* 2001). Therefore RABV and both LBV and DUVV were used in the respective RFFIT assays. The LBV isolate used as a challenge virus was LBVAfrica1999, the DUVV isolate was DUVV2006 and the RABV representative was the challenge virus standard-11 (CVS-11).

2.2.4. Viral genome detection

2.2.4.1. RNA extraction

RNA extraction was performed using TriZol (Invitrogen) and the prescribed protocol with minor modifications. In brief an approximate equivalent of 250 mg of brain material was added to 750 µl of TriZol reagent (Invitrogen) in a 1.5 ml eppendorf tube. This mixture was homogenised by pipetting and thoroughly vortexed and then briefly centrifuged at 3000 x g for five seconds to spin down all contents. To this 150 µl of chloroform (Merck) was added to the sample solution, vortexed and then incubated at room temperature for 10 minutes. After incubation the solution was centrifuged at 12,000 x g at four °C for 10 minutes, to allow phase separation. The aqueous phase was then transferred to a new eppendorf tube and 0.5 ml isopropyl

alcohol (Merck) was added before incubation at room temperature for another 10 minutes. The solution was then centrifuged at 12,000 x g at four °C for another 10 minutes. The aqueous phase was then removed by pipette and discarded. The remaining pellet was washed with one millilitre of 75 % ethanol and centrifugation at 7,500 x g for five minutes at four °C. The liquid phase was removed and the wash step repeated. After the second wash the aqueous phase was removed by pipette and the RNA pellet was allowed to air dry, before the pellet was re-suspended in 25 µl of nuclease-free water (Promega) and incubated at 55 °C for 10 minutes. The re-suspended RNA was checked to confirm concentrations above 25 µg/µl then stored at -20 °C until further use.

2.2.4.2. Quantative Real-time Reverse-Transcription Polymerase Chain Reaction

For the detection of African associated lyssaviruses the amplification of a 126 bp fragment of the nucleoprotein gene was achieved with the use of a 5’ nuclease fluorescent probe and a quantative real-time reverse-transcription PCR as described by Coertse, *et al.,* 2010 with minor modifications to optimise the system for the use with TaqMan Fast Virus 1-step master mix (Applied Biosystems).

In brief; a master mixture was prepared in a 1.5 ml eppendorf tube by adding 9.7 µl of nuclease-free water (Promega), five µl Applied biosystems Master Mix, two µl 541lysfor forward primer [10 pmol] (5’-CACMGSNAAYTAYAARACNAA-3’), two µl 550B reverse primer [10 pmol] (5’-GTRCTCCARTTAGCRCACAT-3’) and 0.3 µl Lyssaprobe620 [10 pmol] (5’-

FAM-CATCACACCTTGATGACAACTCACAA-BHQ-10-3') for a total of 19 µl per sample. After gently mixing the master mix and briefly centrifuging to spin the contents down, 19 µl of the master mix was added to each of the glass capillaries (Roche). For each sample one µl of the re-suspended RNA was added to the respective capillary, additionally one µl of nuclease-free water (Promega) was added to a single separate capillary as a no template control, and one µl of CVS-11 standard RNA was added to another separate capillary as a positive control. All the capillaries were capped using the capillary capping tool (Roche Diagnostics) and centrifuged at 600 x g for three seconds, before being transferred to the LightCycler 1.5 thermocycler (Roche Diagnostics).

Thermocycling conditions for the qRT-PCR were set up as follows: one cycle of reverse transcription with; one step of 50 °C for 30 minutes and one step of 95 °C for five minutes. Then 40 cycles of PCR for quantification with; the first step of 95 °C for five seconds, then a step at 42 °C for 15 seconds with a single acquisition mode and a final step of 72 °C for six seconds with a decreased ramp rate of two. The final cooling cycle was 40 °C for 30 seconds. Upon completion of the thermocycling quantification analysis was performed using LightCycler® software version 4.1.

2.2.4.3. Reverse-Transcription Polymerase Chain Reaction

For samples that tested positive by means of the qRT-PCR, a two-step reverse transcription PCR was performed using AMV Reverse transcriptase (Roche Molecular diagnostics) for the production of cDNA. A five microlitre aliquot of the extracted RNA from section 2.2.4.1. was used as the template

for the cDNA synthesis. To this template one µl of either 550B or 001lysf [10 pmol] primer (Markotter, *et al.,* 2006a) was added for the forward or reverse reaction respectively before the solution was heat shocked at 94 °C for one minute and then placed on ice for five minutes. Simultaneously a master mixture was prepared by the addition of 6.5 µl of nuclease-free water (Promega), 4.5 µl of five times AMV incubation buffer (250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; five mM dithioerythritol; pH 8.5), 2.2 µl [10 mM] dNTPs, 0.4 µl [20 U/µl] AMV reverse transcriptase (Roche Diagnostics) and 0.4 µl [40 U/µl] RNase inhibitor (Roche Diagnostics) per sample. After the template and primer had been on ice for five minutes, 14 µl of the master mix was added and the sample was incubated at 42 °C for 90 minutes on the Thermocycler (GeneAmp® PCR System 2700, Applied Biosystems).

Once the synthesis of the cDNA was completed, the 20 µl reaction was used as the template material for the subsequent PCR. A master mix was prepared with 10 µl Dream Taq buffer [5 x] (Fermentas), 0.25 µl Dream Taq [1.25 U/µl] (Fermentas), one µl forward primer [10 pmol] (either 550B for nested product or 001lysf for full gene length), 1.25 µl reverse primer [10 pmol] (541lys for nested product or 304B for full gene length respectively) and 67.5 µl nuclease-free water (Promega) for each sample. Eighty microlitres of this master mix was added to each of the samples before the following cycling conditions were followed on the Thermocycler (GeneAmp® PCR System 2700, Applied Biosystems). Each sample experienced an initial denaturation step of 94 °C for two minutes, then 40 cycles of 94 °C for 30 seconds, 37 °C for 30 seconds and 72 ˚C for 90 seconds and a final elongation of 72 °C for seven minutes before samples were held at four °C until further processing.

2.2.4.4. Agarose gel electrophoresis

Products from both PCRs were analysed by means of agarose gel electrophoresis. The samples from the 550B and 541lysfor primer set (Coertse, *et al.,* 2011), from the real-time PCR, with an expected fragment size of 140 bp were analysed by means of two percent agarose gel, whilst the fragments of approximately 1,400 bp from the conventional PCR using 001Lysf and 304B primer set were run on a one percent agarose gel using one times TAE buffer (1.6 M Tris-acetate, 40 mM EDTA). Gels were prepared to a total volume of 40 ml with the addition of four µl ethidium bromide [10 mg/ml] before the agarose set. A 100 bp DNA molecular weight ladder (Promega) was loaded on each gel to indicate size of fragments. The gels were run at 120 Volts with a Hoefer power station PS500X (Hoefer). After electrophoresis the gels were photographed under ultraviolet light.

2.2.4.5. Purification of PCR products

The Promega, Wizard® SV Gel and PCR Clean-Up System was used in accordance with the manufacturer's instructions. In brief, the bands were excised from the agarose gel after electrophoresis by means of a sterile scalpel blade and transferred into a sterile 1.5 ml eppendorf tube, which had been pre-weighed. The tube containing the excised band was then reweighed and the weight of the agarose (SeaKem LE agarose, Lonza) gel determined. The membrane wash solution was prepared by adding the indicated volume of 95 % ethanol (Merck) to the stock solution. Membrane binding solution was added to each of the agarose gel containing tubes in a ratio of 10 µl of membrane binding solution per 10 mg of agarose gel. The tube was then

vortexed vigorously and incubated at between 50 and 65 °C for 10 minutes or until the agarose gel had completely dissolved. The tube containing the dissolved agarose gel was briefly centrifuged 8,000 x g for two seconds at room temperature to ensure accumulation of the solution at the bottom of the tube.

For each dissolved agarose gel solution a SV minicolumn was inserted into a sterile collection tube. The dissolved agarose gel solution was transferred to the corresponding SV minicolumn and collection tube assembly by means of pipette and allowed to stand at room temperature for one minute. The aforementioned assembly was then centrifuged at 16,000 x g for one minute. After the initial centrifugation the assemblies were removed, the SV minicolumns were transferred to new collection tubes, whilst the initial tubes with the sample flow through were discarded. The new assemblies then underwent a wash process by the addition of 700 µl of the prepared membrane wash solution to each of the SV minicolumns. The assemblies were then centrifuged at 16,000 x g for one minute again. At the end of this centrifugation the flow through was discarded from the collection tube and the wash step repeated on the assemblies. However, this second wash step was performed with 500 µl membrane wash solution and the assemblies were centrifuged for five minutes at 16,000 x g. The flow through in the collection tubes was discarded and the assemblies underwent a one minute centrifugation at 16,000 x g to facilitate the evaporation of any residual ethanol. After this the collection tubes were disposed of and the SV minicolumns were transferred to new sterile 1.5 ml eppendorf tubes. To each SV minicolumn 25 µl of nuclease-free water (Promega) was added and

allowed to stand for one minute at room temperature. The SV minicolumn and eppendorf tube assembly was then centrifuged at 16,000 x g for one minute. The SV minicolumns were then discarded, whilst the DNA eluent containing eppendorf tubes were stored at -20 °C.

2.2.4.6. Agarose gel electrophoresis

The now purified PCR amplicons were analysed by means of one percent agarose gel (SeaKem LE agarose, Lonza) and electrophoresis as described in section 2.2.4.4.

2.2.4.7. DNA sequencing

DNA sequencing of the purified PCR products was performed with the use of the BigDye Terminator v3.1 Kit (Applied Biosystems, 2002) with minor modifications. In brief; one µl of the five times sequencing buffer, one µl of 3.2 pmol primer 001Lys for the forward reaction and 304B for the reverse sequences) (Markotter, *et al.,* 2006a), two µl of 2.5 x BigDye Terminator mix v3.1 (Applied Biosystems, 2002) and four µl of nuclease-free water (Promega) were added to two µl of the template DNA (adjusted to concentrations between 20 and 100 ng/µl) in individual PCR reaction tubes followed by a two second centrifugation at 8,000 x g to ensure all of the mixture was accumulated at the bottom of the tube. Each 10 µl reaction was loaded into a Thermocycler (GeneAmp® PCR System 2700, Applied Biosystems) and cycled under the following conditions; an initial denaturation step of 94 °C for one minute, followed by 25 cycles of denaturation heating at 94 °C for 10 seconds, annealing at 50 °C for five seconds and extension at 60 °C for four

minutes and finally held at four °C until the samples were removed to be stored at -20 °C.

2.2.4.8. DNA sequencing reaction purification

For each of the 10 µl reaction mixtures a purification process was performed after the completion of the thermocycling. This purification process as described by Applied Biosystems, 2002, involved the addition of one µl of the 125 mM EDTA, one µl of three M sodium acetate and 25 µl of 100 % ethanol (Merck) to each reaction tube containing the 10 µl sequencing product. The solution was briefly mixed by manual agitation and then allowed to incubate at room temperature for 15 minutes. After incubation the solution was centrifuged at 12,000 x g for 20 to 30 minutes and the resulting supernatant was removed by pipette. One hundred µl of 70 % ethanol (Merck) was then immediately added to each reaction tube and centrifuged at 12,000 x g for 10 to 15 minutes. The supernatant was once again removed by pipette and the wash step was repeated. After removal of the supernatant from the second wash step the sample was allowed to air-dry with the lid of the reaction tube open at room temperature for 20 minutes or alternatively placed at 94 °C for one minute. The resulting precipitated reactions were submitted for processing on an ABI 3100 DNA sequencer (AE Applied Biosystems) at the sequencing facility of the Faculty of Natural and Agricultural Sciences of the University of Pretoria, South Africa.

2.2.4.9. Phylogenetic analyses

Sequences obtained from the sequencing facility were initially assembled using the CLC Main Workbench v6 (CLCBio). Forward and reverse sequences of the respective amplicons were assembled to form an accurate consensus sequence. The completed consensus sequence was subsequently exported to BioEdit (Hall, 1999) to allow the assembly of a multiple alignment using the ClustalW function (Hall, 1999; Thompson, *et al.,* 2002).The assembly was constructed from GenBank available sequences representative of all the known lyssavirus species (Table 2.1). A neighbour-joining phylogenetic tree using the Kimura-2 parameter model and bootstrap support of a 1000 replications in MEGA v5.0 (Tamura et al., 2011) was constructed.

Table 2.1. *Lyssavirus* species with corresponding GenBank accession numbers used in the formation of the phylogenetic analysis.

Specie	GenBank accession number	Geographic area of isolation
Australian bat lyssavirus	AF081020	Australia
Australian bat lyssavirus	AF418014	Australia
Australian bat lyssavirus	NC_003243	Australia
Aravan virus	EF614259	Kyrgyzstan
Duvenhage virus	JN986749	Kenya
Duvenhage virus	EU293119	South Africa
Duvenhage virus	EU293120	South Africa
Duvenhage virus	EU623444	South Africa
European bat lyssavirus 1	EU293112	France
European bat lyssavirus 1	EU293109	France
European bat lyssavirus 1	EF157976	Germany
European bat lyssavirus 2	EU293114	Netherlands
European bat lyssavirus 2	EF157977	United Kingdom
European bat lyssavirus 2	NC_009528	United Kingdom
Irkut virus	EF614260	Russia
Khujand virus	EF614261	Tajikistan
Lagos bat virus	EF547447	northern Africa
Lagos bat virus	EF547449	Central African Republic
Lagos bat virus	EU259198	Kenya
Lagos bat virus	GU170202	Kenya
Lagos bat virus	EF547451	South Africa
Lagos bat virus	EF547458	South Africa
Lagos bat virus	EF547453	South Africa
Lagos bat virus	EU293110	Nigeria
Lagos bat virus	EF547459	Nigeria
Lagos bat virus	EF547456	South Africa
Lagos bat virus	EF547455	South Africa
Lagos bat virus	EU293108	Senegal
Lagos bat virus	EF547450	Zimbabwe
Mokola virus	Y09762	Nigeria
Mokola virus	EU293117	Cameroon
Mokola virus	EU293118	Central African Republic
Rabies virus	EU293116	Argentina
Rabies virus	EU293115	France
Rabies virus	AY956319	India
Rabies virus	GU992322	Morocco
Rabies virus	AY705373	United States of America
Rabies virus	EU293111	Thailand
Shimoni bat virus	GU170201	Kenya
West Caucasian bat virus	EF614258	Russia
Ikoma lyssavirus	JX193798	Tanzania
Bokeloh bat lyssavirus	JF311903	Germany

2.2.4.10. Virus Isolation

Virus was isolated from original bat brain (Webster & Casey, 1996) with minor modifications. In brief, approximately 10 % weight per volume suspension of brain sample and DMEM growth media (Lonza) was made. The suspension was then centrifuged at 500 x g for 10 minutes and the supernatant removed. A volume of 500 µl of the supernatant was then added to an approximate 6 x 10^6 mouse neuroblastoma (MNA) cells freshly suspended in DMEM (Lonza) in a 15 ml falcon tube (Greiner bio-one). This was then incubated for 90 minutes at 37 °C and five percent CO_2, with manual agitation every 15 minutes. The incubated suspension was then centrifuged for five minutes at 500 x g and the supernatant discarded. The resulting cell pellet was re-suspended in six ml of modified DMEM (Lonza). One and a half millilitres of the re-suspended cells were used to seed a T25 tissue culture flask (Greiner bio-one).

Additionally an eight well, six mm Cel-Line slide (Thermo Fisher Scientific) was prepared as outlined in section 2.2.3.2. with seven µl of the re-suspended virus containing supernatant being added to seven µl of modified DMEM and 14 µl of MNA cells per well excluding the cell culture control well. Both the T25 flask and the slide were then incubated for 48 hours at 37 °C and five percent CO_2. After incubation the Cel-Line slide had its' supernatant decanted and the slide was prepared for florescent staining as described in section 2.2.3.2. The slides were then read under 160 to 200 times magnification with a fluorescent microscope (Carl Zeiss Axiovert 25 CFL) and 20 microscope fields per well were examined for the presence of fluorescence indicating virus growth.

2.2.4.11. RNA extraction

With the completion of viral isolation, RNA was extracted from the cell culture to confirm that the virus being maintained was the same as that originally identified. The protocol used for this process was the same as the TriZol (Invitrogen) protocol described in section 2.2.4.1. except that 500 µl of cell culture supernatant was used rather than 250 µg equivalent of brain material. Protocols as described in sections 2.2.4.3-5. were followed to perform RT-PCR, agarose electrophoresis, sequencing and purification resulting in a nucleoprotein gene sequence that could be compared to the original sequence.

2.3. Results

2.3.1. Serological assay

For the determination of the neutralising antibody presence in South African bats, serum samples from 324 bats covering 16 genera and 28 species (Table 2.2.) were subjected to separate RFFITs for LBV, DUVV and RABV. An additional 26 bat samples were subjected to DUVV RFFITs only. These blood samples were collected from bats throughout South Africa from 2007 until the end of 2012. Due to the possibility of samples producing a level of background neutralisation and the majority of samples that produced neutralising responses doing such for more than one of the challenge viruses, a cut off dilution of 1:5 was deemed to be a negative response and thus only when the challenge viruses could be neutralised at dilutions of 1:25 and greater were they considered positive neutralising against that virus. From the total samples tested, 107 individual samples produced neutralising results against LBV (95 % confidence 0.2798-0.3847). A further 44 samples were able to neutralise DUVV (95 % confidence 0.0937-0.1661) whilst only seven samples were able to neutralise RABV (95 % confidence 0, 0095-0.0459). These samples only achieved this to a low level of 1:25 (Table 2.2. and Appendix S1), whilst 84 samples were able to produced neutralising antibodies to more than one of the challenge viruses and some dilution (Appendix S14).

Table 2.2. Summary of bat samples screened by both quantitative real-time polymerase chain reaction (qRT-PCR) and rapid fluorescent focus inhibition test (RFFIT) assays.

Genus	Specie	no. of positive samples\no. tested from:			
		Brains by QRT-PCR	Serum by RFFIT		
			LBV	DUVV	RABV
Chaerephon	*ansorgei*	0\2	0\2	0\2	0\2
	pumilus	0\19	3\7	3\7	0\7
Epomophorus	*spp.*	0\1	0	0	0
	gambianus	0\3	1\3	0\3	0\3
	wahlbergi	0\32	3\4	0\5	0\4
Eptesicus	*hottentotus*	0\1	0\2	1\2	0\2
Glauconycteris	*variegatus*	0\6	1\5	2\5	0\5
Hipposideros	*caffer*	0\4	1\2	1\2	0\2
Kerivoula	agentata	0\1	0	0	0
Miniopterus	*spp.*	0\34	0	0	0
	natalensis	0\31	9\52	6\52	0\52
	schreibersii	0\8	0	0	0
Molossid	*spp.*	0\2	0\1	0\1	0\1
Mops	*condylurus*	0\10	1\6	0\6	0\6
	midas	0\1	0	0	0
Myotis	*spp.*	0\1	0	0	0
	tricolor	0\1	0\1	0\1	0\1
	welwitschii	0\1	0	0	0
Neoromicia	*capensis*	0\16	0\4	0\10	0\4
	helios	0\5	0\3	1\3	0\3
	nana	0\14	1\6	0\7	0\6
	rueppellii	0\1	0\1	0\1	0\1
	zuluensis	0\4	0\2	1\2	0\2
Nycticeinops	*schlieffenii*	0\13	0\5	1\5	0\5
Nycteris	*thebaica*	1\18	5\12	6\13	1\12
Otomops	*spp.*	0\2	0	0	0
	martiensseni	0\5	0	0	0

Pipestrullus	*spp.*	0\3	0	0	0
	kuhli	0\1	0	0	0
	hesperidus	0	3\6	1\6	0\6
	rusticus	0\7	0\4	4\4	1\4
Rhinolophus	*spp.*	0\7	0\0	0\4	0\0
	capensis	0\1	0	0	0
	clivosus	0\7	0	0	0
	darlingi	0\6	0\3	0\5	0\3
	denti	0\5	0\2	1\4	0\2
	fumigatus	0\2	0\1	0\1	0\1
	hildebriandti	0\3	0	0	0
	landeri	0\1	0\1	0\1	0\1
	simulator	0\11	0\5	1\6	0\5
Rousettus	*aegyptiacus*	0\16	67\129	7\129	2\129
Sauromys	*petrophilus*	0\1	0\0	0\1	0\0
Scotophilus	*spp.*	0\21	4\16	4\16	2\16
	dinganii	0\37	5\23	2\23	0\23
	leucogaster	0\6	2\4	1\4	0\4
	nigrita	0\1	0\1	0\1	0\1
	viridis	0\8	0\3	0\3	0\3
Tadarida	*aegyptiacus*	0	0\3	0\4	0\3
Taphozous	*mauritianus*	0\4	0\4	0\4	0\4
Unknown Chiroptera		0\7	1\2	1\6	1\2
Unknown Insectivorous bat		0\21	0	0	0
Unknown Vespertilionidae		0\2	0\1	0\1	0\1
Total		413	324	350	324
		1\413	107\324	44\350	7\324

From the 350 individual samples, two groups could be identified that required a more intensive investigation. The two groups were comprised of the 52 samples of *Miniopterus natalensis* from the Irene cave (Gauteng) and the 129 *Rousettus aegyptiacus* sampled from the Matlapitsi cave population in Limpopo (Table 2.1 and Appendix S1). The Irene population had an 11.5 % sero prevalence for DUVV neutralising antibodies with six of the 52 individual samples being able to neutralise DUVV in excess of a 1:25 dilution (95 % confidence intervals 0.0478-0.2413). The more in-depth investigation of this population also shows that an approximate 17 % (95 % confidence intervals 0.0869-0.3082) of the population were able to neutralize LBV above the threshold limit of 1:5 and those 11 individuals (42 %) showed some low level of neutralisation against more than one of the challenge viruses. Similarly, for the group of *R. aegyptiacus* (Limpopo province), nearly 52 % (95 % confidence intervals 0.4301-0.6075) (67 of the 129) of the sampled population were capable of neutralising LBV above the threshold limit, whilst only five percent (95 % confidence intervals 0.024-0.1128) were able to neutralise DUVV and two percent (95 % confidence intervals 0.0027-0.0605) able to neutralise RABV challenge virus (Table 2.1.).

2.3.2. Viral RNA extraction

To determine whether lyssavirus genomic RNA was present in South African bat populations, 413 (Table 2.2.) individual brain samples from South African bats collected from nine major regions throughout South Africa were screened by means of the quantative real-time PCR (Figure 2.1.). The

samples represented 21 genera and 39 confirmed species of South African bats. All but one of the total RNA extractions produced in this study yielded negative results for the amplification of the 126 bp region of the N-gene of the lyssavirus genome. The majority of the samples processed were obtained from apparently healthy bats that were actively flying and foraging before capture so there is the possibility of some bias being demonstrated from these data.

Of the 413 individual bats tested for the presence of lyssavirus RNA, the only sample to produce a positive result was UP 1540, a *Nycteris thebaica* from the Rooiberg region of the Limpopo province of South Africa in 2012. After confirmation of viral infection in the sample by means of FAT, RNA extraction and sequencing resulted in the production of the 1412 bp N-gene (GenBank accession number: KC86630) sequence for this virus. Phylogenetic analysis demonstrated that the infection was caused by Duvenhage virus (Figure 2.2), with 98.3 to 99.3 % sequence similarities to the other South African Duvenhage isolates as demonstrated in the pair wise distance analysis (Table 2.3.).

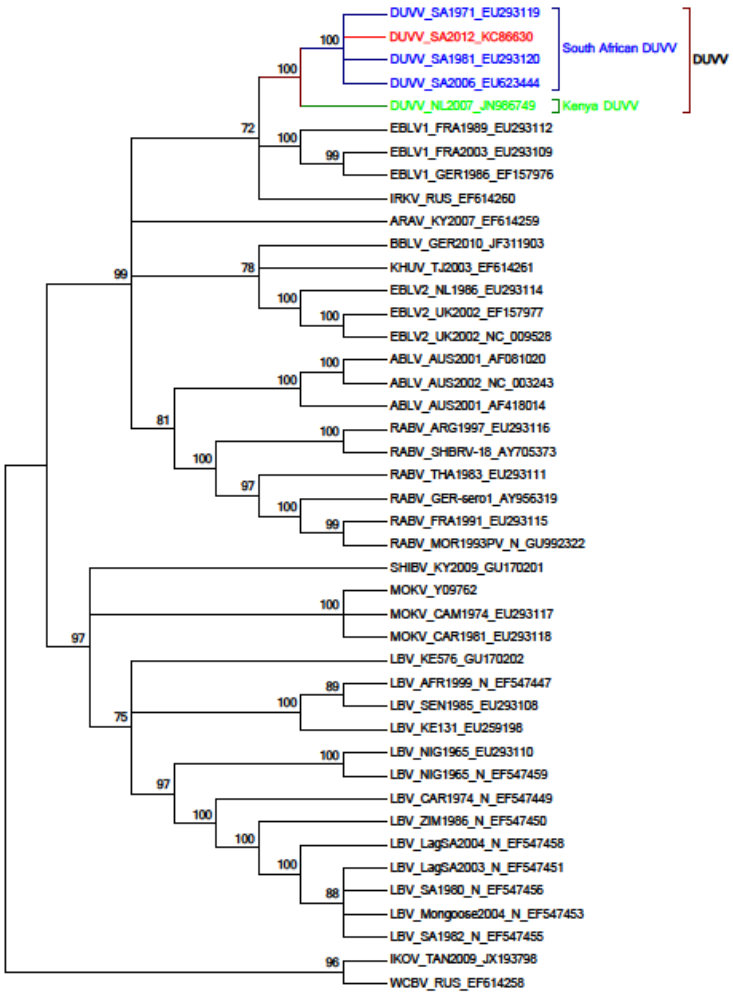

Figure 2.2. Phylogenetic tree analysis demonstrating the neighbour-joining relationship between the new viral isolate (DUVV SA2012) and other lyssavirus sequences.

Table 2.3. Pair wise distances between the new DUVV isolate (DUVV SA2012) and lyssavirus reference sequences as determined using the Kimura 2-parameter model.

Virus	NL2007	SA1971	SA1981	SA2006
DUVV_NL2007_JN986749				
DUVV_SA1971_EU293119	0.085			
DUVV_SA1981_EU293120	0.091	0.009		
DUVV_SA2006_EU623444	0.093	0.010	0.015	
DUVV_SA2012_KC86630	0.088	0.006	0.014	0.014

2.4. Discussion

There were two core aspects to this study. The first was the serological surveillance of the meta-population of the bats in South Africa for the presence of lyssavirus neutralising antibodies whilst the second aimed to identify viral infections. Towards this first aim the serum of several bat species were collected throughout South Africa by means of both passive and active surveillance programs. The passive process relied upon the submission of the more metropolitan associated species by the public whilst the active programs allowed for targeting of previously implicated species and areas of increased biodiversity. This generalised approach to serological surveillance allowed for the initial assumptions of which viruses were circulating within the bat populations. Although the presence of neutralising antibodies does not definitively identify a viral specie, it does indicate the presence of viruses closely related to those used in the various assays.

Prior to this study, *Epomophorus wahlbergi* from the coastal areas of Kwa-Zulu Natal (Markotter, *et al.,* 2006b), was the only bat species that had been examined for the presence of LBV within South Africa. Whilst in the West of Africa Hayman *et al.,* 2008, had provided evidence of LBV neutralisation in three other African fruit bats (*Eidolon helvum, Ep. gambianus* and *Ep. buettikoferi*). The results of this surveillance not only confirm that some sampled members of *Ep. wahlbergi* possessed neutralising antibodies against LBV but also that members of 15 other species of South African bats (*Chaerephon pumilus, Ep. gambianus, Eptesicus hottentotus, Glauconycteris variegatus, Hipposideros caffer, Miniopterus natalensis, Mops condylurus, Neoromicia nana, Nycteris thebaica, Pipistrellus hesperidus, Rhinolophus darlingi, R. simulator, Rousettus aegyptiacus, Scotophilus dinganii and S. leucogaster,*) were able to neutralise LBV challenge virus.

This expansion of species capable to neutralise LBV, brings to light the possibility of two scenarios regarding this group of lyssaviruses. Either the number of species affected by LBV in South Africa is far more extensive than just the historically implicated fruit bats, in which case the number of potential contacts and spill-over events could be greatly increased. In the other scenario, it might not be LBV, but cross-neutralising viruses that circulated within these populations. This possibility also raises concern for potential contacts but would further provide evidence to the greater diversity, should this be proven by virus isolation. The results obtained in this study with the insectivorous bats displaying up to 19 % sero prevalence whilst the larger colony of African fruit bat, *R. aegyptiacus* providing up to 52 % seroprevelance, somewhat mirrors the prevalence described by Hayman *et al.,* 2008 with *E. helvum* and *Ep. buettikoferi* displaying 33 % and 37 % respectively and *Ep. gambianus* only displaying 3 % neutralisation. Suggesting that LBV is not uniformly distributed amongst the different species populations.

Whilst the numbers of individuals sampled and screened for DUVV neutralising capability was essentially the same, a far lower proportion of animals demonstrated these antibodies however there were still representatives from 17 species that did (*Chaerephon pumilus, Eptesicus hottentotus, Glauconycteris variegatus, Hipposideros caffer, Miniopterus natalensis, Neoromicia helios, N. rusticus, N. zuluensis, Nycticeinops schlieffenii, Nycteris thebaica, Pipistrellus rusticus, Pipistrellus hesperidus, P. rusticus, Rhinolophus simulator, Rousettus aegyptiacus, Scotophilus dinganii and S. leucogaster).* These species capable of neutralising DUVV confirm both of the historically implicated species, *Nycteris thebaica* and *Miniopterus natalensis* as coming into contact with this virus. The remaining 15 species displaying neutralisation are predominantly insectivorous species, with only a few *Rousettus aegyptiacus* from the northern parts of South Africa representing the fruit bats. The numbers of *R. aegyptiacus* displaying DUVV neutralising antibodies was far less than those demonstrating LBV neutralisation, suggesting that this virus is less prominent within the large aggregations and may possibly be more a case of ‘spill over’ from the insectivorous species sharing the cave system.

The numbers of individuals sampled for the study display some bias towards the *R. aegyptiacus* from the Limpopo province of South Africa, due to the active surveillance being undertaken on a large colony. This bias can however be negated if the number of species affected is considered rather than the number of individuals affected per specie. From the samples collected, covering the North and East of the country (Figure 2.1) it is evident that both LBV and DUVV are more extensively present than was previously thought. It is significant to note that despite the bias in sampling locations, the majority of species shown to be sero positive can be found dispersed throughout the rest of the country, so the seroprevelance demonstrated from this work should give a fair indication for the country as a whole.

The two populations that were examined more extensively, presented two different scenarios, with the *M. natalensis* showing sero positive for only one of the challenge virus per individual, whilst the majority of *R. aegyptiacus* demonstrating neutralisation capable antibodies against DUVV or RABV were also able to neutralise LBV. With the *M. natalensis* samples having been collected on a monthly basis throughout the course of the study, rather than in two major collections as with *R. aegyptiacus*, it is possible that the *R. aegyptiacus* were sampled during the peak of sero prevalence of LBV which overshadows any other lyssavirus neutralising antibodies, whilst the sampled *M. natalensis* display a more robust model of the colony.

The seven sampled bats that produced a level a neutralisation against RABV were from four identified species *Nycteris thebaica, Pipistrellus rusticus, R. aegyptiacus* and two *Scotophilus spp.* that are still in the process of being assigned to a species, and an unidentified species. Six of these samples produced low results whilst the remaining *R. aegyptiacus* produced an intermediate response, this low level neutralisation coupled with no historical records of RABV infection nor sero prevalence within African bat species suggest that these results are more likely caused by a cross reacting virus. It is however noteworthy to mention that the *R. aegyptiacus* demonstrating the intermediate level of neutralisation did not demonstrate the ability to neutralise either of the other two challenge viruses, removing DUVV as a possible cross neutralising cause in this case, but further screening will be required to draw light onto this situation.

With 33.0 %, 12.6 % and 2.2 % neutralisation against LBV, DUVV and RABV respectively from the samples screened it is highly suggestive that the South African bat populations come into far more contact with LBV like viruses. This may indicate that the distribution of LBV is far more extensive than the old world fruit bats or that there are viruses able to produce cross neutralising antibodies against LBV circulating among these bat populations.

From the results reported in this chapter it has been shown that the primary hypothesis that lyssaviruses are circulating within the South African bat populations is completely valid. The second portion of this research chapter focused on the detection of viral RNA and the subsequent isolation of the virus. This portion of the study was achieved with the use of the highly sensitive molecular technique of qRT-PCR, this system provided the best use of the extremely limited quantities of sample and would allow for isolation and sequencing of any samples that resulted to be positive. This system is also far more sensitive than the traditional fluorescent antibody test and can detect viral presence down to single copy numbers despite remaining specific for lyssavirus detection. The degeneracies incorporated into the qRT-PCR probe and primers allows for the detection of all African associated lyssaviruses and provides enough variation that new viruses within the genus should be detected by this technique. All qRT-PCR positives were confirmed by traditional FAT and if results were found to be in conflict, RNA extraction, qRT-PCR and sequencing were performed on the sample again to exclude the possibility of contamination. This system achieved a single positive sample from a sample size of 413 bats (0.24 %). A recent second isolation was not included within this study (scope of the study for degree purposes). The second positive sample and subsequent isolate was obtained after the commencement of the thesis preparation, but the sample has been identified as LBV from an *Ep. Wahlbergi*. Sequencing of the nucleoprotein was still on going at the time of submission. This low percentage of virus positive individuals is most likely due to the fact that the majority of the tested samples, 351 of the 413 apparently healthy animals, were captured during free flight, with no signs of illness. In contrast both the cases that have yielded virus have been from individuals submitted by the public, displaying unusual behaviour, in hindsight

most plausibly due to the resulting encephalitic infection. Although there may be high seroprevelance of these viruses in these bats, actual viral isolation is relatively uncommon.

The isolated virus was a new representative of the DUVV specie, most closely related to the other South African isolates, in particular the 1971 virus. The close identity (0.6 % difference) to the 1971 South African isolate, despite a huge time gap (41 years) between isolations, suggests that both of these viruses are part of the same continuous (and stable) DUVV cycle that has been occurring in this geographic region. This isolation represents the second from *N. thebaica* in this region of the continent, strengthening the possibility that this bat species is a reservoir host or otherwise involved with the cycling of this virus. Indeed, this species has been overlooked in the past, with preferential focus given to *M. natalensis*. The implication of *N. thebaica* could provide future problems of exposures as these bats can be brought into closer contact with humans due to use of night roosts. Notwithstanding these two isolations from *N. thebaica*, the mechanism of maintenance within these bats is still unclear and will require further study. The numbers of individual bats present within roosts appearing to be insufficient for what is expected for non-volant associated lyssaviruses. The possibility of a more persistent form of viral maintance within the bat populations should be entertained. However, *N. thebaica* commonly share cave roosts with *M. natalensis* in this region which do exist in colonies of appropriate size for lyssavirus maintenance. Taken with the finding that a relatively large proportion of sampled *M. natalensis* possessed DUVV neutralising antibodies, it is possible that *M. natalensis* could be a primary host whilst *N. thebaica* are unfortunate casualties of spill-over events.

The serological results show that active viral circulation may be far more extensive than previously thought. Species previously overlooked or under sampled, were found to have high levels of neutralising antibodies against either LBV or DUVV. These bats are either host to these viruses or viruses similar enough to provide cross neutralising antibodies. Apart from the evidence of viral exposure, viral infection was not proven as yet and confirmatory isolations may be achieved from symptomatic individuals in the future.

Chapter 3

DNA barcoding of South African bat species

3.1. Introduction

DNA barcoding is a revolutionary molecular technique applied to assign a unique genetically determined identification to the species under investigation. This designation is determined by analysing the divergence present in a standardised gene region. Since Hebert *et al*., 2003, a segment near the 5' end of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene has predominantly been used for classifying members of the animal kingdom (Clare, *et al.,* 2011). The use of barcoding lends itself to the process of species identification and discovery, with differences becoming visible at a genetic level that may have been otherwise undetectable. Despite these benefits the use of barcoding for mammals had been limited to isolated studies however recently with the increase of habitat loss the process is gaining favour (Clare, *et al.,* 2006; Clare, *et al.,* 2011; Francis, *et al.,* 2010). The ability to detect subtle differences between individuals provides a useful tool in surveillance programs fighting disease. Barcoding has been put to use in identifying wild bird species affected by avian influenza allowing predictions of the disease spread to be plotted with migration patterns (Lee, *et al.,* 2010), and has been used to identify disease carrying mosquitoes in India (Kumar, *et al.,* 2007). These scenarios highlight the need to understand an affected host in order to form contingencies against the disease and the barcoding process can have a critical role to play.

The order of chiropteran lends itself to the use of the barcoding mediated identification process, making up around 20 % of the known mammal species (1116 of 5416 species) (Clare, *et al.,* 2006; Clare, *et al.,* 2011) many of which are small and nondescript, leaving identification up to the use of dated morphology keys (Monadjem, *et al.,* 2013). Studies of bat populations have been limited until recently and the studies performed have occasionally relied on other gene regions such as the cytochrome b (Baker & Bradley, 2006) or the NADH dehydrogenase protein of the mitochondrial subunit 1 (Mayer & von Helversen, 2001). Fortunately the cytochrome b and COI regions appear to undergo similar levels of selective pressure and can be roughly compared (Clare, *et al.,* 2011). However with the need to standardise the process the COI has found more favour. With the current number of sub-Saharan described chiropteran species already exceeding 130 and new species identified regularly (Fenton, 2013) there is a need of a reliable and standardised technique for detection and identification. Historically the use of field guides and detailed tables of morphologically accurate measurements and distinguishing features coupled with the experience of a taxonomist has been the only answer to this dilemma. However, the process of visual identification and measurement comparison is time consuming and susceptible to error with many related species providing overlapping values with one another. The limited use of sight in bats also places into doubt the selective pressures placed upon morphological characteristics (Clare, *et al.,* 2006; Simmons & Voss, 1998).

For these above mentioned reasons and the lack of time typically afforded to the actual process of allocating species identification to individual samples

processed in the field, the majority of associated work is following the trend of using DNA barcoding in an attempt to innovate the field of study and eliminate the human bias or errors. In contrast to the methodical approach of referring forearm length, scull length, measurements and the dentition of the individual to vast keys, DNA barcoding theoretically provides a sequence specific to a specie.

To address this lack of information currently available for the some 116 recognised bat species in the southern Africa region, there is the need to develop an extensive data base of the complete DNA barcode linked to morphologically confirmed specimens, preferably museum voucher specimens. This would create a reliable set of genomic sequences and morphological confirmed vouchers for comparison of samples needing to be assigned to a species and the possible delimitation of new future specimens with morphological information. For the production of this initial data base it was decided to use the universal primers of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) as described by Folmer *et al.,* 1994. These primers are positioned some 710 bp apart on most genomes, resulting in good quality reads in excess of 500 bp, sufficient for assigning 117 specimens to their 27 representative species.

3.2. Materials and Methods

3.2.1. Sample collection

The bat samples used for DNA barcoding were collected as described in Section 2.1.1. From the individuals taken as museum vouchers for morphological identification, tissue samples of either pectoral muscle or a

wing punch were used for DNA extraction. Of the samples taken 117 (Appendix S2) individual specimens produced successful sequence representing 27 species from 18 genera (Table 3.1.).

Table 3.1. Representative numbers of bat species used for the production of COI DNA barcoding sequences.

Species name	Number of individuals barcoded
Chaerephon pumilus	4
Epomophorus gambianus	5
Epomophorus wahlbergi	10
Eptesicus hottentotus	2
Hipposideros caffer	2
Miniopterus natalensis	2
Mops condylurus	5
Neoromicia capensis	12
Neoromicia helios	1
Neoromicia nana	3
Neoromicia rueppellii	1
Neoromicia zuluensis	1
Nycteris thebaica	5
Nycticeinops schlieffenii	7
Otomops martiensseni	1
Pipistrellus hesperidus	4
Pipistrellus rusticus	6
Rhinolophus darlingi	3
Rhinolophus denti	3
Rousettus aegyptiacus	5
Sauromys petrophilus	1
Scotophilus dinganii	13
Scotophilus leucogaster	6
Scotophilus nigrita	1
Scotophilus viridis	7
Tadarida aegyptiaca	4
Taphozous mauritianus	3
Total:	117

3.2.2. Morphological identification

Morphological identification of voucher specimens was performed by Dr. Teresa Kearney of The Ditsong National Museum of Natural History, (formerly Transvaal Museum), Pretoria, South Africa as described in section 2.2.2.

3.2.3. DNA extraction

Genomic DNA was extracted either from 'wing punches' of captured and released bats or pieces of pectoral muscle (less than 25 mg) from the museum voucher specimens. The DNeasy® Blood & Tissue Kit (Qiagen) was used following the manufacturer's guidelines for animal tissues with minor modifications. Before the procedure was started the addition of the appropriate amount of 100 % ethanol (Merck) to the concentrated buffers, AW1 and AW2 was undertaken as instructed. The pectoral muscle or piece of wing was added to a 1.5 ml sterile microtube (Qiagen) to which 180 µl of ATL Buffer (Qiagen) was added. To this solution 20 µl of Proteinase K solution (Qiagen) was added before the mixture was vortexed and incubated at 56 °C for three to four hours or until the sample was completely dissolved. During this incubation the sample was briefly vortexed every hour to assist in the process. Upon visual confirmation of the completely dissolved sample, the solution was vortexed for 15 seconds. A volume of 200 µl of AL Buffer was then added to the sample and mixed thoroughly by vortexing before a ten minute incubation at 56 °C. Finally 200 µl of 100 % ethanol (Merck) was added to each of the solutions and vortexed again.

The homogenised sample solution was then transferred by pipette to a DNeasy Mini spin column (Qiagen) in a two ml collection tube. The DNeasy Mini spin column and collection tube assembly was centrifuged at approximately 4,300 x g for one minute. The DNeasy Mini spin column was transferred to a new collection tube, whilst the flow through and initial collection tube was discarded. Five hundred µl of AW1 Buffer were then added to the new assembly and centrifuged at 4,300 x g for one minute. Once again the flow through and collection tube was disposed of, whilst the DNeasy Mini spin column was transferred to a new collection tube. To this new assembly, 500 µl of AW2 Buffer was added before centrifugation for three minutes at 13,150 x g. The flow through and collection tube was discarded and the DNeasy Mini spin column moved to a new sterile 1.5 ml microtube. For the elution of the product, 100 µl of AE Buffer was added to the assembly and allowed to stand at room temperature for one minute before centrifugation at 4,300 x g for one minute. An additional 100 µl AE buffer was added to the assembly and allowed to stand for one minute before centrifuging was repeated at 4,300 x g for one minute. The eluted product was stored at -20 °C until later use.

3.2.4. Agarose gel electrophoresis

Products from the DNA extraction were analysed by agarose gel electrophoresis. A one percent agarose gel (SeaKem LE agarose, Lonza) was prepared by adding 0.4 grams of agarose powder (SeaKem LE agarose, Lonza) to a volume of 40 ml one times TAE buffer (1.6mM Tris-acetate, 40mM EDTA). Before solidification of the gel, four µl of ethidium bromide [10 mg/ml]

was added to facilitate visualisation of genomic material. Electrophoresis was performed at 120 volts for 30 minutes (ENDURO^{TM}; Labnet), after which the gels were photographed under ultraviolet light.

3.2.5. Polymerase chain reaction

The polymerase chain reaction (PCR) for the amplification of the approximately 710 bp fragment of the COI region was performed on the extracted total DNA. The primers used for the purpose of this reaction were FPLMERLC01490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and FOLMERHCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer, *et al.,* 1994). For each sample a total volume of 50 µl PCR reaction mixture was prepared: 27.25 µl nuclease-free water (Promega, Madison, USA), five µl Dreamtaq buffer [5 x] (Fermentas), two µl FPLMERLC01490 [10 pmol], two µl FOLMERHCO2198 [10 pmol], two µl mixed dNTPs [10 mM], 1.5 µl $MgCl_2$, 0.25 µl [5 U/µl] Dreamtaq (Fermentas) and 10 µl of template DNA. Each PCR reaction tube was transferred to a Thermocycler (GeneAmp® PCR System 2700, Applied Biosystems) with the following temperature cycling; initial single denaturation step at 94 °C for five minutes, followed by 25 cycles of a 94 °C denaturation for one minute, a 48 °C annealing for one minute and a 72 °C elongation for 90 seconds and a final elongation step of 72 °C for 10 minutes was performed before the reaction was stored at four °C.

3.2.6. Agarose gel electrophoresis

PCR products were analysed by means of one percent agarose (SeaKem LE agarose, Lonza) gel and electrophoresis, as described above in

section 3.2.4. A 100 bp DNA molecular weight marker (Promega) was included to confirm the size of the PCR amplicons produced.

3.2.7. Purification of PCR products

The Promega, Wizard® SV Gel and PCR Clean-Up System was used in accordance with the manufacturer's instructions as described in section 2.2.4.5.

3.2.8. Agarose gel electrophoresis

The now purified PCR amplicons were again analysed by means of one percent agarose gel (SeaKem LE agarose, Lonza) and electrophoresis as described in section 3.2.4.

3.2.9. DNA sequencing

DNA sequencing of the purified PCR products was performed with the use of the BigDye Terminator v3.1 Kit (Applied Biosystems, 2002) with minor modifications as described in section 2.2.4.7. One microlitre of 3.2 pmol primer (FPLMERLC01490 (5'-GGTCAACAAATCATAAAGATATTGG-3') for the forward sequence reactions and FOLMERHCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') for the reverse sequences) were used.

3.2.10. DNA sequencing reaction purification

For each of the 10 µl reaction mixtures a purification process was performed after the completion of the thermocycling as explained in section

2.2.4.8. The resulting precipitated reactions were submitted for processing on an ABI 3100 DNA sequencer (AE Applied Biosystems) at the sequencing facility of the Faculty of Natural and Agricultural Sciences of the University of Pretoria, South Africa.

3.2.11. DNA sequencing analysis

The raw sequence information obtained from the sequencing facility were imported into CLC Main workbench 6 (CLCBio) for annotation and manipulation. Where possible forward and reverse sequences of the same sample were used to create a consensus sequence. Published GenBank sequences (Table 3.2.) and a COI reference sequence from the Barcode Of Life Database (BOLD) were used to ensure the sequence included the appropriate region of the mitochondrial genome (originally positions 1490 and 2198 of the *Drosophila yakuba;* honeybee 5' nucleotide GenBank accession number, X03240) (Folmer *et al.,* 1994) and was of acceptable length (500 bp to 680 bp of the total 710 bp). The edited consensus sequences were exported to Bioedit v7, where a multiple alignment was produced using the ClustalW accessory (Hall, 1999). The multiple alignment was then imported into MEGA v5.0 (Tamura, *et al.,* 2011) for the construction of a neighbour-joining phylogenetic tree using the Kimura-2 parameter model and bootstrap support of a 1000 replications.

In the neighbour-joining phylogenetic analysis, samples that were not separated by bootstrap values greater than 70 were considered to be members of the same clade. Kimura-2 parameter sequence divergences for all genera were determined using the pair wise distances in MEGA v5.0.

Species that split into two or more groups in the phylogenetic tree with bootstrap support above 70 and displayed a sequence divergence greater than 2.5 % are hypothesized to represent provisional species or lineages, warranting further investigation (Clare, *et al.,* 2006; Clare, *et al.,* 2011).

Table 3.2. Species and associated accession numbers (GenBank or BOLD) of the representative bat sequences used for phylogenetic analysis.

Specie	Accession number
Chaerephon plicatus	BOLD_AAK0536
Epomophorus gambianus	BOLD_AAC2947
Epomophorus wahlbergi	GenBank_ JF442398.1
Eptesicus brunneus	BOLD_AAF2882
Glauconycteris beatrix	BOLD_AAE0467
Glauconycteris poensis	BOLD_AAF5438
Hipposideros caffer	BOLD_AAX1242
Miniopterus natalensis	GenBank_ JF442530.1
Miniopterus schreibersii	BOLD_AAC3658
Mops mops	GenBank_BM510-04
Myotis nattereri	BOLD_ACB3797
Neoromicia brunneus	GenBank_ JF444136.1
Neoromicia nanus	GenBank_ JF444202.1
Neoromicia nanus	GenBank_ JF444202-1.1
Nycteris grandis	BOLD_AAI3441
Nycteris thebaica	GenBank_ JF442546.1
Otomops martiensseni	GenBank_ JF442564.1
Pipistrellus eisentrauti	BOLD_AAH9463
Pipistrellus nanulus	BOLD_AAD5669
Pipistrellus nanus	BOLD_ABZ1913
Pipistrellus tenuis	BOLD_AAB2554
Rhinolophus alcyone	BOLD_AAD6851
Rhinolophus hipposideros	BOLD_AAD0389
Rhinolophus pumilus	BOLD_AAI0440
Rousettus aegyptiacus	BOLD_AAA2863
Rousettus aegyptiacus	GenBank_JF444434
Scotophilus dinganii	GenBank_ JF442688.1
Scotophilus heathi	BOLD_AAD3569
Scotophilus kuhli	BOLD_AAC0094
Scotophilus kuhli	GenBank_HM541937
Tadarida teniotis	BOLD_AAF7233
Taphozous longimanus	BOLD_AAH9837
Taphozous melanopogon	GenBank_ABRVN431-06

3.3. Results

To determine the suitability of the COI region of South African bats for the purposes of DNA barcoding, sequence for 117 individual samples collected from several locations throughout South Africa (Figure 2.1.) were produced. DNA sequences produced in this study cover 18 genera and 27 separate species based on morphological classification (Table 3.1.). For the purposes of this study all sequences produced were longer than 500 bp in length and represented the region selected by the Folmer *et al.,* (1994) primer set. All of the barcode sequences produced within this study, clustered into their respective genera as indicated by morphological classification, with strong bootstrap values in excess of 70, separating the different genera (Figure 3.1.). The 15 sequences representing *Epomophorus spp*., (Figure 3.1 and 3.2.) form a clade separate from other bat genera, with two distinct lineages. However these lineages are not species specific with members of the sampled *E. wahlbergi* appearing in both groups, with a minimum difference of 5.7 % between the lineages whilst the inter lineage difference is between one and two percent (Appendix S3).

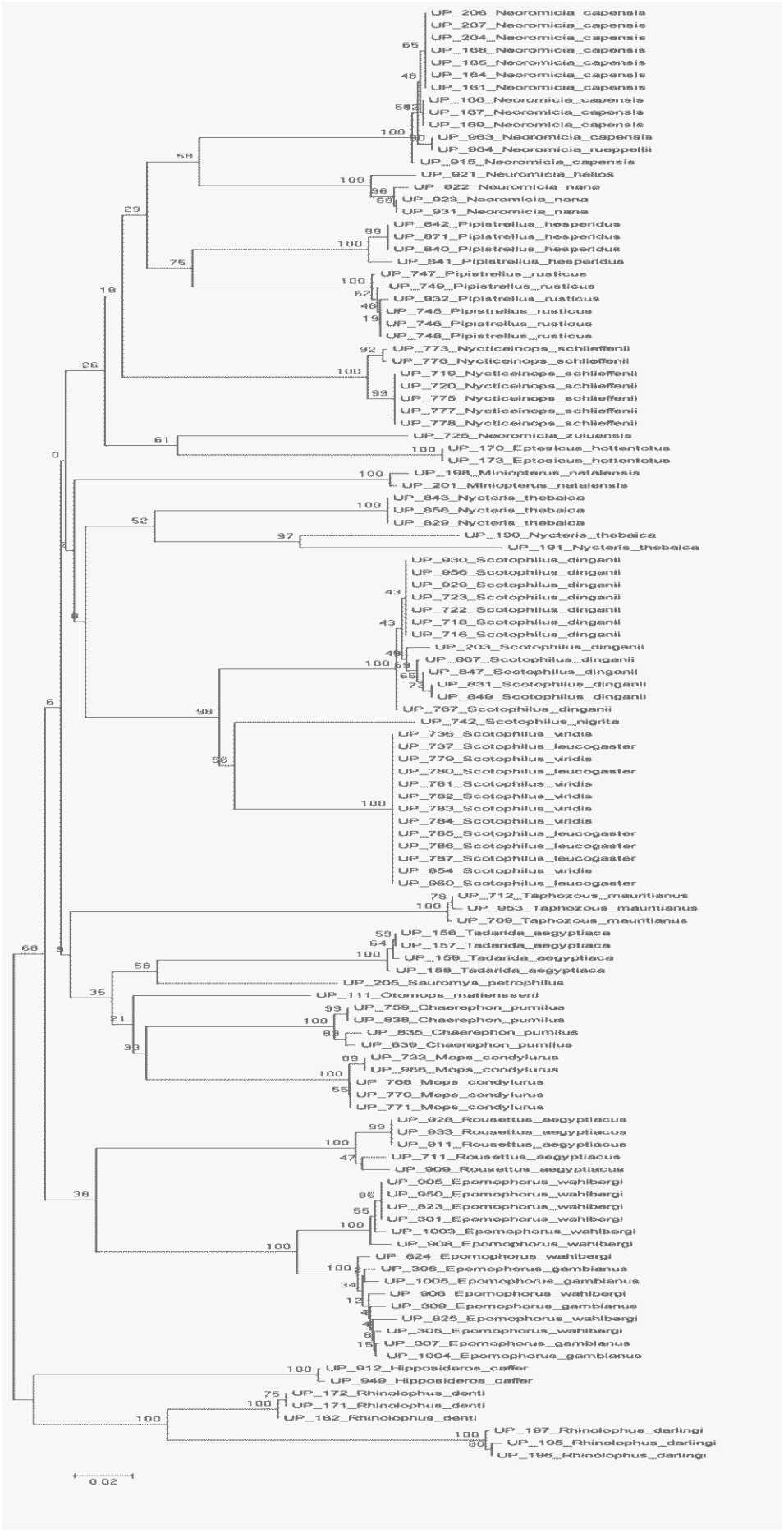

Figure 3.1. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of 27 bat species from South Africa. With four *Chaerephon pumilus,* five *Epomophorus gambianus*, 10 *Ep. wahlbergi,* two *Eptesicus hottentotus,* two *Hipposideros caffer*, two *Miniopterus natalensis*, five *Mops condylurus,* 12 *Neoromicia capensis*, one *Neoromicia helios*, three *Neoromicia nana,* one *Neoromicia rueppellii,* one *Neoromicia zuluensis*, five *Nycteris thebaica*, seven *Nycticeinops schlieffenii,* one *Otomops martiensseni,* four *Pipistrellus hesperidus*, six *P. rusticus,* three *Rhinolophus darlingi,* three *R. denti,* five *Rousettus aegyptiacus,* one *Sauromys petrophilus,* 13 *Scotophilus dinganii,* six *S. leucogaster,* one *S. nigrita,* seven *S. viridis,* four *Tadarida aegyptiaca* and three *Taphozous mauritianus.*

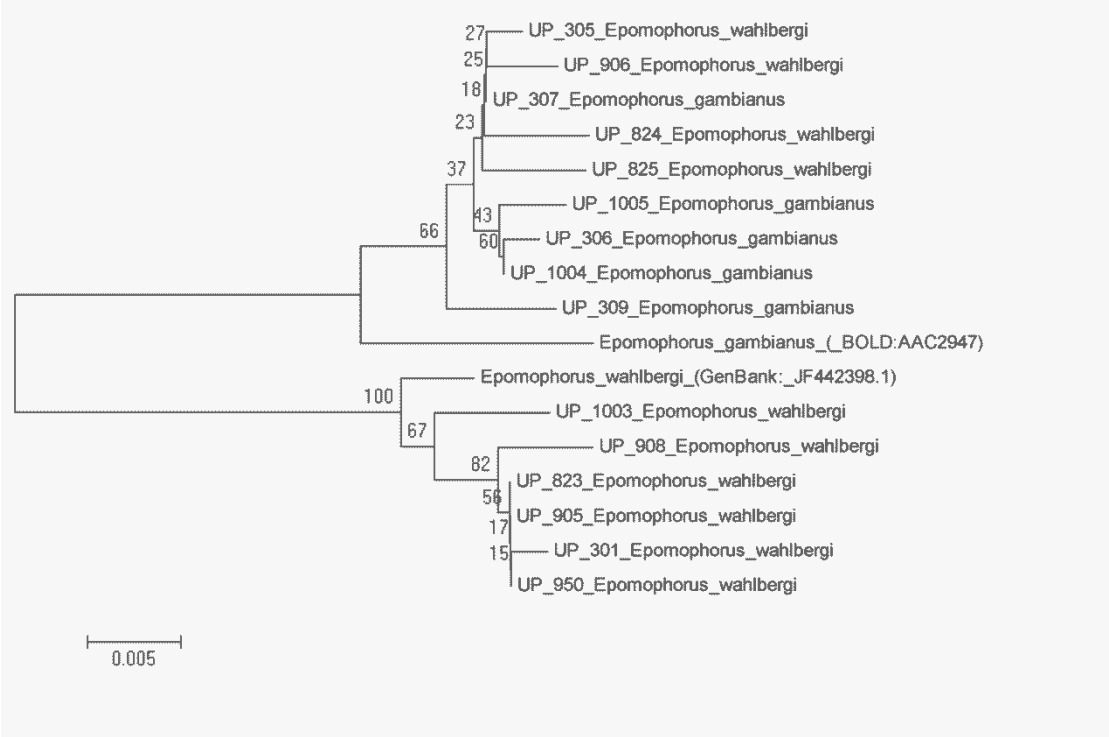

Figure 3.2. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the five *Epomophorus gambianus* and 10 *Ep. wahlbergi*. samples from South Africa.

The *Rousettus aegyptiacus* clade (Figure 3.1. and 3.3) made up of five samples is clearly separate from the other bat species, yet there are also signs of divergence within the produced sequences (Figure 3.3.). The sequence divergence seen (Appendix S4) shows a difference of 2.5 - 2.7 % between the two lineages whilst differences within each lineage are only between 0.2 – 1.8 %.

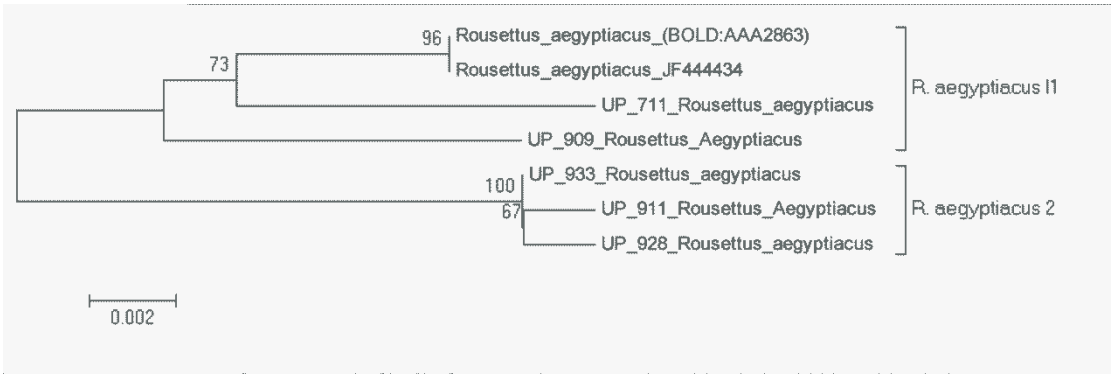

Figure 3.3. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the five *Rousettus aegyptiacus* samples from South Africa.

The 27 sampled *Scotophilus spp.* covering four species form a strong clade divergent from other genera (Figure 3.1 and 3.4.). The species of *S. leucogaster* and *S. viridis* form a single lineage within this clade with sequence differences between zero and one percent of the two species, whilst *S. dinganii* and *S. nigrita* are visibly separate with up to 14 % difference between members of these species.

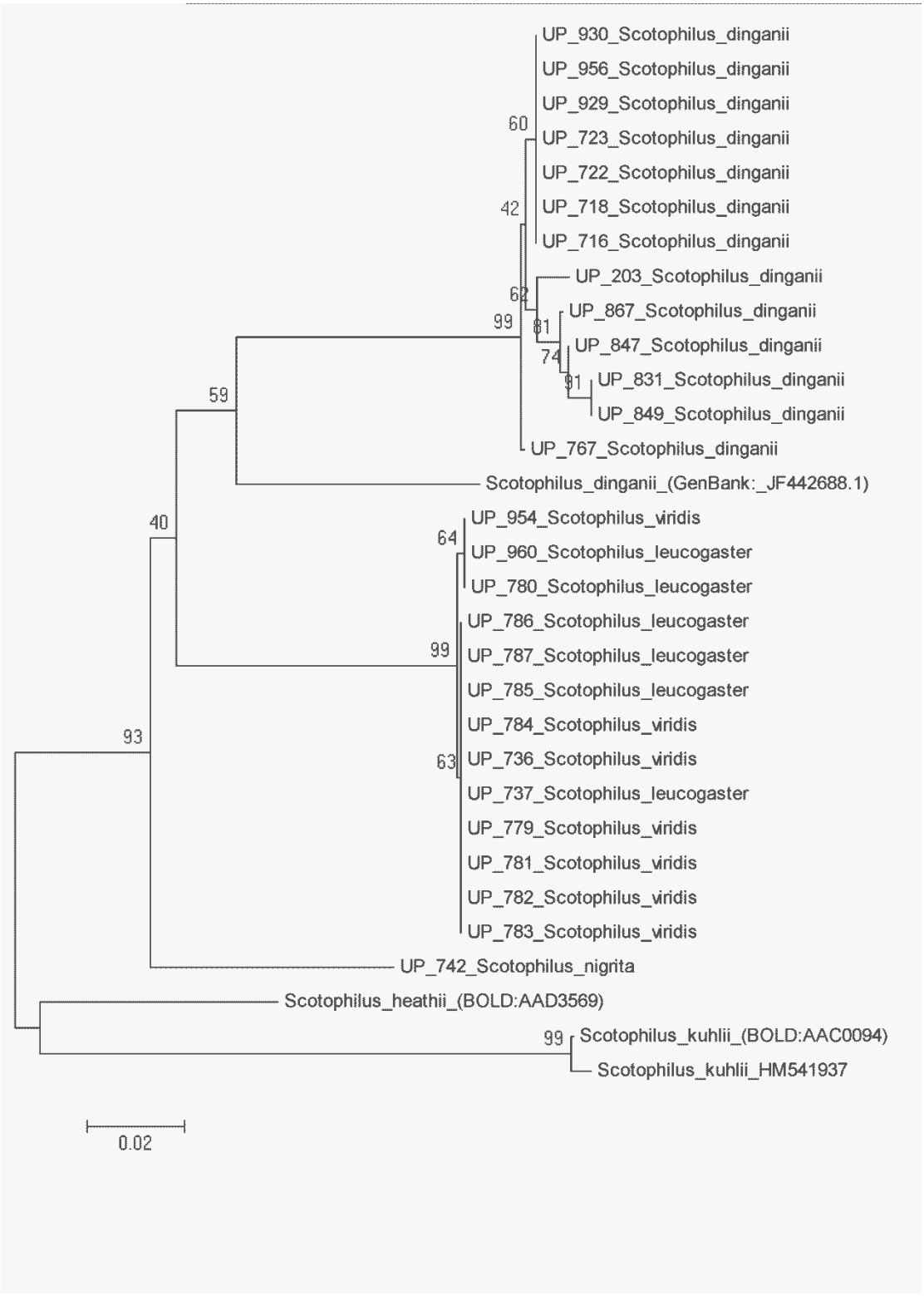

Figure 3.4. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the 13 *Scotophilus dinganii,* six *S. leucogaster,* one *S. nigrita* and seven *S. viridis,* samples from South Africa.

The five representatives of *Nycteris thebaica* (Figure 3.1 and 3.5.) form two lineages strongly separated from the GenBank sequence used as reference sequence for the specie. The lineage of three samples separates at 4.7 % difference from the reference and between 17 and 20 % difference from the other lineage of two samples whilst maintaining a low intra lineage difference (Appendix S5).

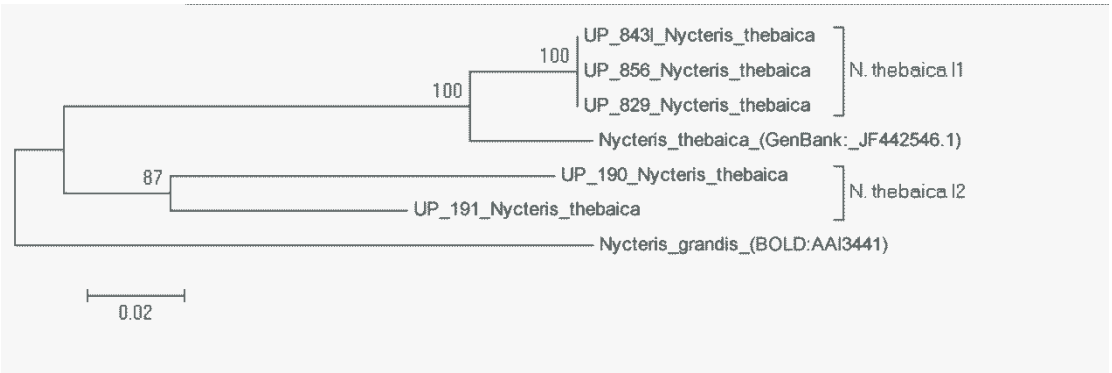

Figure 3.5. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the five *Nycteris thebaica* samples from South Africa.

The 18 samples of *Neoromicia* spp. (Figure 3.1 and 3.6.) provide varying results with the 12 representatives of *N. capensis* forming a single lineage with intra species differences of only 0.2 % whilst the other six members representing the other four species display inter species differences of up to 28 % with intra species differences of *N. helios* between 1.3 and 4.9 % (Appendix S8).

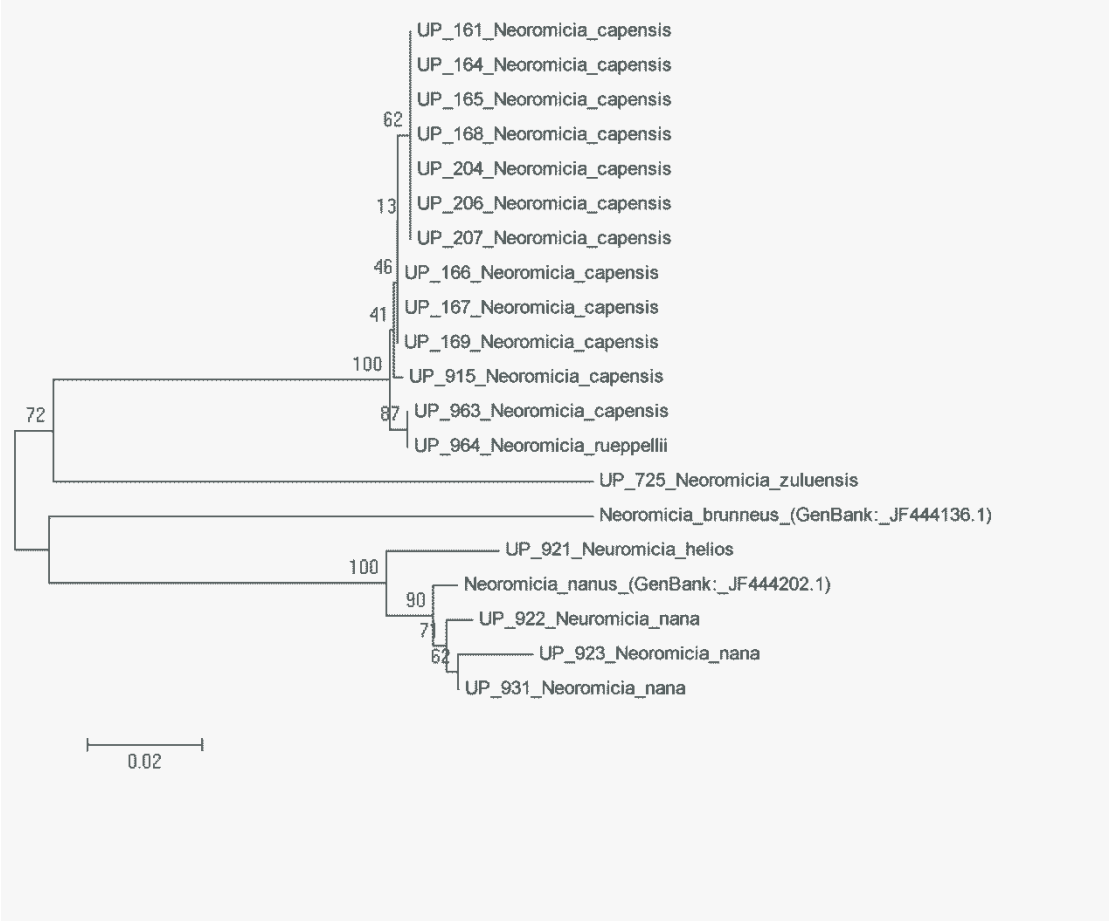

Figure 3.6. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the 12 *Neoromicia capensis,* one *Neoromicia helios,* three *Neoromicia nana,* one *Neoromicia rueppellii,* one *Neoromicia zuluensis* samples from South Africa.

The ten representatives of *Pipistrellus* separate into their two respective species with intra species differences of 1.7 % for *P. hesperidus* and 0.7 % for *P. rusticus* with an intra species difference of up to 15 % (Appendix S9).

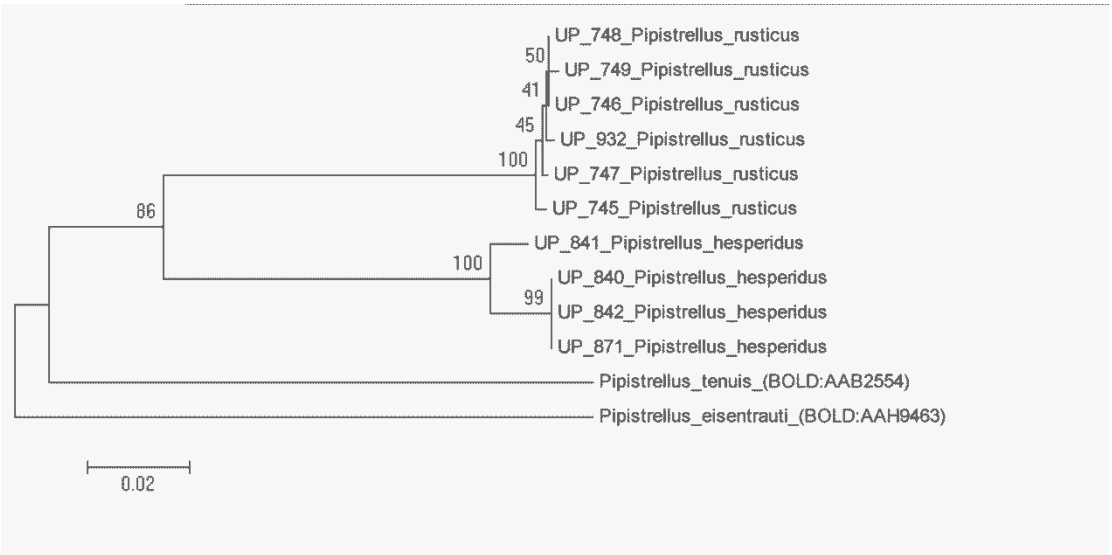

Figure 3.7. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the four *Pipistrellus hesperidus* and six *P. rusticus* samples from South Africa.

Rhinolophus species provided intra species differences of up to 0.4 % whist intra species differences were between 14 and 22 % (Appendix S09).

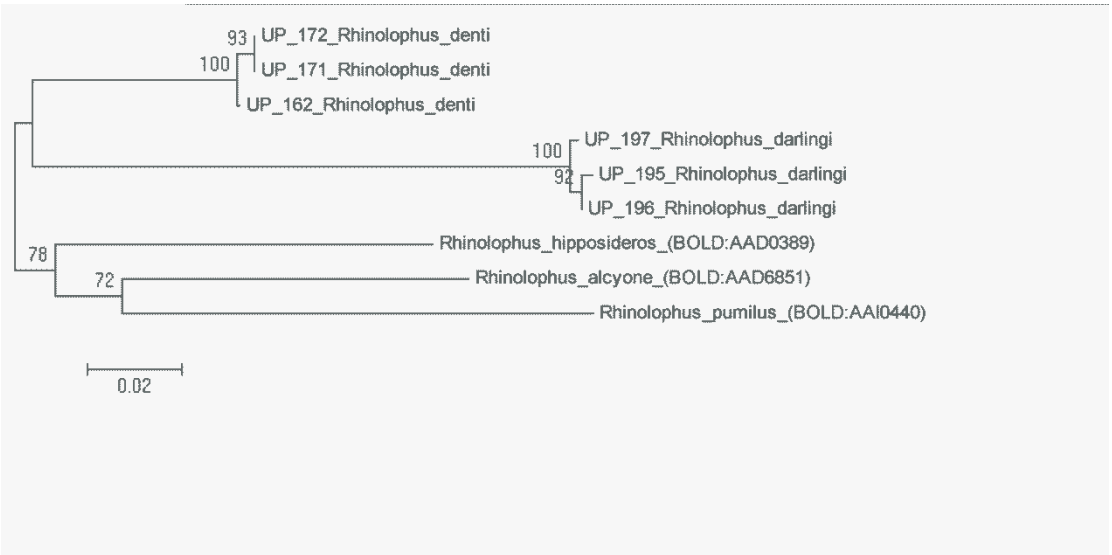

Figure 3.8. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the three *Rhinolophus darlingi* and three *R. denti* samples from South Africa.

Intra species differences of up to 0.8 % were produced for *Taphozous mauritianus* (Figure 3.1, 3.9 and Appendix S11).

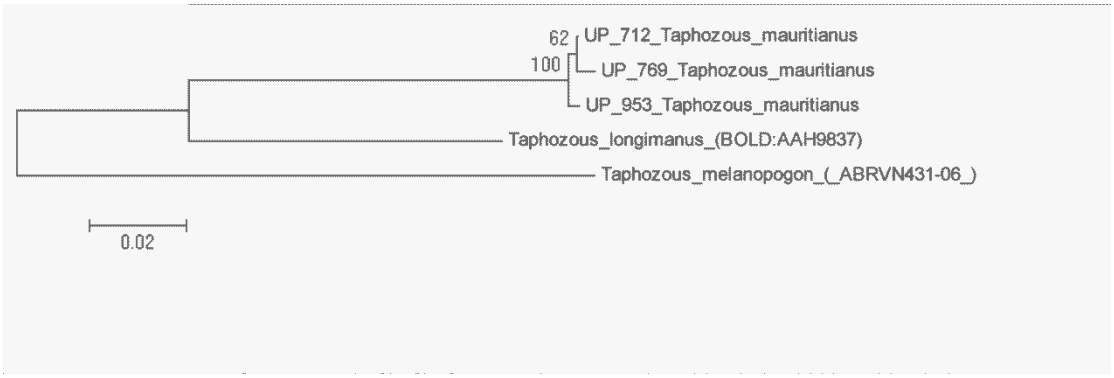

Figure 3.9. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the three *Taphozous mauritianus* samples from South Africa.

Tadarida aegyptiaca samples had differences of up to 0.4 % (Figure 3.1, 3.10 and Appendix S11).

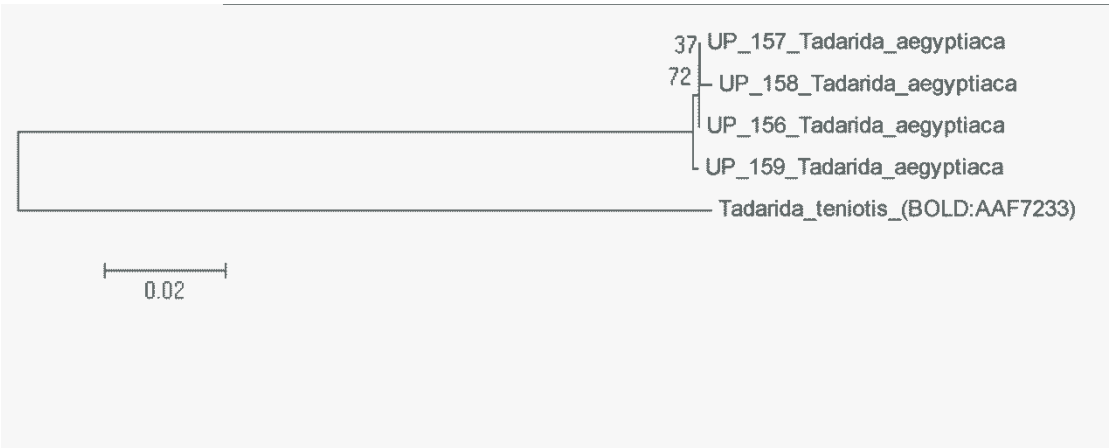

Figure 3.10. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the four *Tadarida aegyptiaca* samples from South Africa.

The seven samples of *Nycticeinops schlieffenii* (Figure 3.1 and 3.11) show signs of possible non-geographical divergence within their clade, no differences in lineage 1, 0.3 % difference within lineage two and a difference of between 1.7 and 2.1 % between the lineages.

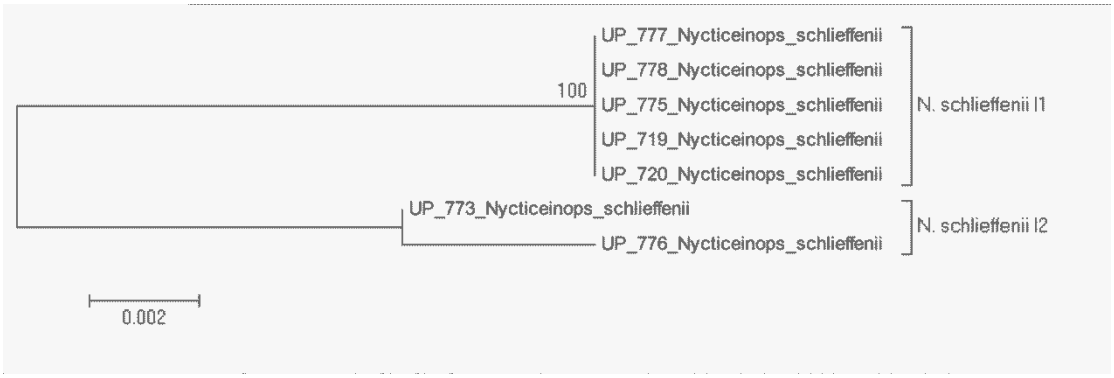

Figure 3.11. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the seven *Nycticeinops schlieffenii* samples from South Africa.

The four samples of *Chaerephon pumilus* had sequence differences of less than 1.3 %. The *Miniopterus* samples provided sequence differences of more than 10 % from the reference sequences (Appendix S13) whilst the *Hipposideros caffer* sample UP 0948 produced an intra species difference of 23 % (Figure 3.12.; Appendix S13). The other samples produced intra species differences of less than one percent with an average intra species difference of 17 %.

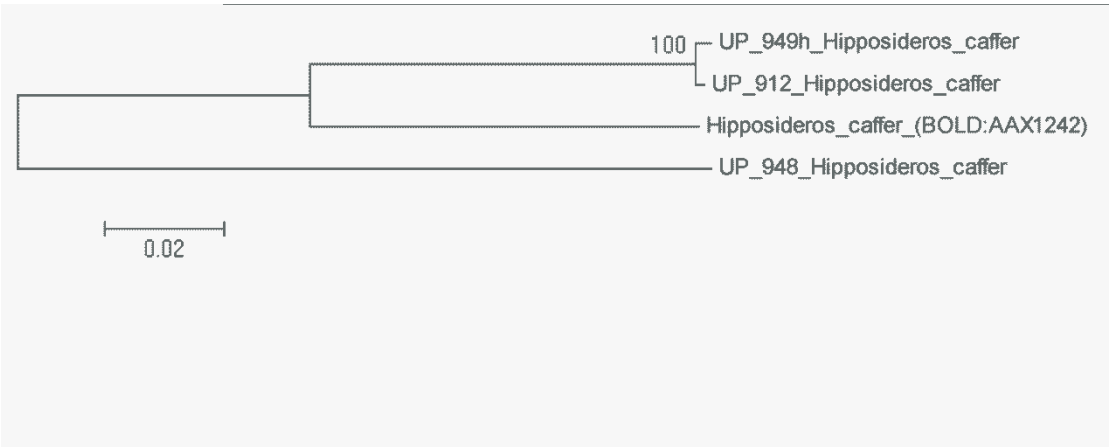

Figure 3.12. Neighbour-joining phylogenetic tree of COI sequence divergences (Kimura-2 parameter) of the three *Hipposideros caffer* samples from South Africa.

3.4. Discussion

This chapter of the study focused on providing usable and accurate sequence data for the purpose of providing DNA barcodes for morphologically identified South African bat species. The results obtained lend strong support to the hypothesis that the COI region is an appropriate target for this purpose. The sequences produced in this study are amongst the first of their kind for South Africa and to our knowledge the only available representative COI barcodes for the majority of these southern African species. This work, in addition with previous and on-going projects is starting to fill in the barcodes of the plethora of African bats which have till recently been somewhat neglected. The partial COI sequence length of 680-710 bp was not obtained for all samples and all sequences below the threshold of 500 bp continuous read length (as suggested by BOLD) were removed from further analysis. The 117 sequences of acceptable length and quality represent 18 genera and 27 separate species of South African bats collected from nine geographical regions within South Africa. The sequences produced all cluster into distinct genetic groups, with clear differentiation between genera, supported by strong bootstrap values in the phylogenetic trees (Figure 3.1.). However, below the genus level of classification, the occurrence of some discrepancies in the groupings of the closely related *Scotophilus leucogaster* and *S. viridis* and between *Epomophorus wahlbergi* and *Ep. gambianus* shows a potential limitation in the use of this specific region of the genome for DNA barcoding. The highly conserved nature of this gene region, which provides the majority of the appeal for a near universal target for eukaryotic life, leaves it susceptible to missing subtle differences between recently diverging species,

that have not been allowed sufficient evolutionary time to develop distinguishing differences in this region (Santamaria, *et al.,* 2007). The genetic group of *Scotophilus spp.* raises an interesting example of the lack of clear resolution between closely related species. Two of the four species sampled, namely *S. dinganii* and *S. nigrita* are sufficiently different to be confidently placed into their own species groups along with the available reference sequences of *S. heathi* and *S. kuhli*. However, the samples of *S. leucogaster* and *S. viridis* form a single genetic group with intra species differences of less than 2.5 % when the COI is analysed. This lack of genetic differentiation between morphologically distinct species reiterates the potential limitation of the system and raises some as yet unanswered questions in the process of bat speciation, which will need further investigation. However, these two species have been at the centre of an on-going species debate since the first descriptions as to whether they are indeed separate species or rather parts of a species complex (Jacobs, *et al.,* 2006). The DNA barcoding seems to strongly favour that these species be combined as a single genetic group. The results produced by the *Epomophorus spp.* samples similarly produce interesting results. As has been seen by all the genera of bats sequenced, the genus is confidently separated from the other bats, but at the species level there is the introduction of confusion. The Species of *E. wahlbergi* and *gambianus* produce two separate clades within the genus however these clades are not mutually exclusive. There are morphologically identified samples of *E. wahlbergi* grouping within the predominantly *E. gambianus* clade and *E. gambianus* samples comfortably grouping with reference sequences of *E. wahlbergi.* Whether this is evidence of genetic

exchange between the two species, these species not being completely separate and rather being possible members of a still diverging group or the lingering evidence of a common ancestor that has been retained differently within pocket populations of the different species will need to be determined by future findings.

The samples of *Miniopterus natalensis, Nycticeinops schlieffenii* and *Rousettus aegyptiacus* present a different situation. Whilst at the genus level all the samples confidently group within their expected localities at the species level some confusion arises. Samples of the same species show evidence of separation into two lineages within that specie, producing the appearance of the initiation of species divergence. This divergence of the clades is further complicated by the fact that the individuals found in these separating groups do not appear to have any distinguishing reason for their separation. Samples collected at the same geographical site and at the same time are found in either grouping. Similarly the separation is not gender specific with both males and females featuring in either of the groups. This apparent separation is most apparent in the *Rousettus aegyptiacus* samples. Whilst this apparent divergence is as yet undetermined for these genera and will warrant future investigation, the separation in the *R. aegyptiacus* could be explained by the presence of at least two major populations of South African *R. aegyptiacus* that although separated genetically due to choice of maternity roost sites, interact with one another during migration and relocation in search of food, maintaining the overall gene flow within the South African meta-population.

The final point of interest to be raised and arguably the most interesting is the genetic story told by the *Nycteris thebaica* samples. Whilst all the

specimens all key to *N. thebaica* using the key in Meester *et al*. (1986), with bifid upper incisors, the same morphology tragi, and forearms of acceptable length for *N. thebaica*. Two individuals from the same geographical location produce sequence as genetically different from the rest of the samples and reference sequences as from *N. grandis.* This genetic data of a difference of 2.1 % strongly suggests that these individuals are member to a different subspecies falling short of the 2.5 % proposed for different species. With such intriguing results, this species is definitely worthy of additional work to find the source of these differences.

From the results produced in this chapter of the study, it is clear that the COI system of DNA barcoding is a good option for the initial identification of South African bat species to at least a genus level. The results show that members of species morphologically identified as a single group will group in general together as a single group from their DNA, meaning that from DNA alone an individual can be aligned to a genus level classification, provided that there are ample, good quality reference samples with which to compare the new sequence. This being said, the results obtained, also far too clearly showed that there are limitations to the system that must be understood to obtain the full benefit available from such an approach. There is also concern on the accuracy of the data obtained from the BOLD network, whilst the DNA information may well be very clear, if there are problems with the morphological identification, the resulting comparisons would be misleading.

Thus in conclusion, the COI DNA barcoding system is a good starting point for the identification of samples. This initial identification will move the sample to a genus level with ease and onto a species level classification in

most cases. This would save countless hours in the identification process and providing a very useful starting point for the less experienced identifier. Barcoding will also provide a useful means to identify a specimen without the need of an entire carcass, allowing sampling and study of endangered or threatened species without the need of taking vouchers from the population and allowing the retrogressive DNA analysis of tissue samples.

Chapter 4

Concluding Remarks

The serological surveillance of South African bat species for two rabies-related viruses previously isolated from South Africa, DUVV and LBV, showed that both the populations of *Rousettus aegyptiacus* and *Miniopterus natalensis* as well as the meta-population of South African bats comes into contact with these viruses or a ones similar enough to producing cross neutralising antibodies. The percentage of neutralisation detected against all the challenge viruses was above expectation and was demonstrated to occur in bat species that have not been previously implicated with lyssavirus cycles. The true extent to which these bat populations are affected will need to be determined by future projects expanding on both the species investigated and the numbers investigated from those species. It will also be of great interest to determine the variation of the serology throughout the course of the year within these affected bat populations, allowing the extent of ecological impact upon viral loads to be examined. Expansion of the panel of challenge viruses to include at least the other African associated viruses, Ikoma lyssavirus, Shimoni bat virus and West Caucasian bat virus, will most likely provide a more accurate reflection of the viral presence within the country and may help to explain the large proportion of samples able to neutralise Lagos bat virus.

The expansion of the surveillance programs would have the added benefit of increasing the odds of obtaining additional viral isolates, which would not only help clarify the viral species circulating in the populations of interest but will be the best opportunity to detect new viral species. As was

demonstrated by this study the conversion from sampled numbers to resulting viral isolation strengthens the thought that these viruses rarely affect bat populations in the same way as their non-volant counterparts. The isolation of the sixth Duvenhage virus provides valuable insight to the genetic diversity, geographical distribution and the possible host species of this virus. Whilst the presence of Lagos bat virus within South African *Ep. wahlbergi* demonstrates the need for continued work focussing on these species. With the increased knowledge in both the species of virus and the potential hosts, this research project has effectively provided evidence of the true extent of these viruses and may in the future help to explain how these viruses are maintained within these host populations.

The level of accuracy displayed by the COI barcoding system for species identification of South African bats in this study provided an essential starting point for the future work. The system consistently displayed reliable differentiation between the species, and highlighted the need for further investigation into the possibility of specie complexes in the groups that varied from their morphological classification. With the continued expansion of the database this system will allow for rapid and accurate species level identification for most and provide substantially reduced list of possibilities for the remainders. An accurate delimitation system such as this can be used as an essential component of disease control in the future, with the ability of sick individuals to be assigned to a species rapidly and thus allowing resources to be focused. This system of DNA barcoding can also help with conservation efforts as morphologically similar groups can display their true divergence and in so doing so help to explain the processes of speciation and evolution.

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Appendix S1. Bat samples collected throughout South Africa that were used for either serological screening against rabies virus, Duvenhage virus and Lagos bat virus or were used for viral RNA detection or both.

UP number	Date collected [dd/mm/yyyy]	*Species names*	Serology	qRT-PCR	Province	Locality
UP 0004	20/08/2004	*Neoromicia nana*	√		Mpumalanga	Laughing waters farm, Nelspruit
UP 0013	26/08/2004	*Rhinolophus spp.*	√		Eastern Cape	Andries Vosloo Kudu Reserve, Grahamstown
UP 0014	26/08/2004	*Rhinolophus spp.*	√		Eastern Cape	Andries Vosloo Kudu Reserve, Grahamstown
UP 0016	26/08/2004	*Rhinolophus spp.*	√		Eastern Cape	Andries Vosloo Kudu Reserve, Grahamstown
UP 0018	26/08/2004	*Rhinolophus spp.*	√		Eastern Cape	Andries Vosloo Kudu Reserve, Grahamstown
UP 0052	22/11/2007	*Scotophilus dinganii*		√	Gauteng	Pretoria
UP 0053	19/06/2007	*Neoromicia capensis*		√	Gauteng	Pretoria
UP 0054	08/08/2007	*Serotine spp.*		√	Gauteng	Pretoria
UP 0055	11/10/2007	*Scotophilus dinganii*		√	Gauteng	Pretoria
UP 0056	02/05/2004	*Nycteris thebaica*		√	Kwa-Zulu Natal	Shongweni valley
UP 0057	20/02/2003	*Otomops martiensseni*		√	Kwa-Zulu Natal	Durban
UP 0058	15/04/2004	*Pipistrellus hesperidares*		√	Kwa-Zulu Natal	Nkandla forest
UP 0059	11/11/2003	*Nycteris Thebaica*		√	Kwa-Zulu Natal	Westville
UP 0060	15/04/2004	*Pipistrellus hesperidares*		√	Kwa-Zulu Natal	Nkandla forest
UP 0061	26/05/2003	*Scotophilus dinganii*		√	Kwa-Zulu Natal	Durban
UP 0063	17/06/2004	*Pipestrullus kuhli*		√	Kwa-Zulu Natal	Amanzimtoti
UP 0064	23/02/2005	*Pipestrullus hesperidares*		√	Kwa-Zulu Natal	Sudwana Bay

UP 0065	15/05/2004	*Rhinolophus darlingi*		√	Kwa-Zulu Natal	Umzinto
UP 0066	22/04/2003	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Pinetown
UP 0067	01/09/2002	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Illovo
UP 0068	19/05/2004	*Scotophilus dinganii*		√	Kwa-Zulu Natal	Durban
UP 0069	05/12/2002	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0070	27/05/2004	*Otomops martiensseni*		√	Kwa-Zulu Natal	Durban
UP 0071	15/05/2005	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Melmoth
UP 0074	25/12/2005	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Chelsen Prep school, Durban
UP 0075	28/07/2005	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Amanzimtoti
UP 0076	23/11/2005	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0077	23/11/2005	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0079	04/04/2003	*Epomophorus wahlbergi*	√	√	Kwa-Zulu Natal	Umdloti
UP 0082	15/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Melmoth
UP 0084	14/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Eshowe
UP 0085	2002	*Scotophilus dinganii*		√	Kwa-Zulu	Durban

					Natal	
UP 0087		*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0098	08/08/2005	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Yellow wood Rd
UP 0099	15/05/2005	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Melmoth
UP 0100	15/05/2005	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Melmoth
UP 0101	2002	*Pipistrellus nanulus*		√	Kwa-Zulu Natal	Durban
UP 0102	15/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Melmoth
UP 0103	15/05/2005	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Melmoth
UP 0104	28/09/2004	*Rhinolophus hildebriandtii*		√	Kwa-Zulu Natal	Mpumalanga
UP 0109	28/09/2004	*Rhinolophus hildebrandtii*		√	Kwa-Zulu Natal	Mpumalanga
UP 0111	17/05/2004	*Otomops martiensseni*		√	Kwa-Zulu Natal	Durban
UP 0114	04/01/2004	*Pipistrellus nanulus*		√	Kwa-Zulu Natal	Durban
UP 0115	14/05/2005	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Entumeni
UP 0116	07/02/2003	*Nycteris thebaica*		√	Kwa-Zulu Natal	Durban
UP 0118	14/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Eshowe

UP 0119	07/02/2003	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Durban
UP 0121	2002	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0124	25/04/2003	*Pipistrellus nanulus*		√	Kwa-Zulu Natal	Umdloti
UP 0125	15/05/2005	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Melmoth
UP 0126	27/02/2004	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Amanzimtoti
UP 0127	17/12/2002	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Eston
UP 0129	15/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Melmoth
UP 0131	14/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Eshowe
UP 0132	25/01/2006	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Durban
UP 0134	04/01/2004	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Durban
UP 0135	Sep-05	*Otomops martiensseni*		√	Kwa-Zulu Natal	Pinetown
UP 0138	15/05/2005	*Rhinolophus clivosus*		√	Kwa-Zulu Natal	Melmoth
UP 0139	Aug-05	*Miniopterus schreibersii*		√	Kwa-Zulu Natal	Durban
UP 0140	25/01/2006	*Otomops spp.*		√	Kwa-Zulu Natal	Durban
UP 0141	25/01/2006	*Otomops spp.*		√	Kwa-Zulu	Durban

					Natal	
UP 0142	2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Allerton
UP 0143	2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Allerton
UP 0144	2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Allerton
UP 0145	17/07/2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0147		*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0148	23/06/2006	*Otomops martiensseni*		√	Kwa-Zulu Natal	Durban
UP 0149	29/05/2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0150	06/06/2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0151	25/03/2006	*Epomophorus wahlbergi*	√	√	Kwa-Zulu Natal	Durban
UP 0152	20/04/2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0153	10/04/2006	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Durban
UP 0154	10/06/2007	*Myotis welwitschii*		√	Mpumalanga	20 km NE of Bronkhorstspruit, Vlakfontein 457 JR
UP 0155	11/01/2006	*Myotis spp.*		√	Mpumalanga	Sudwalaskraal 271 JT
UP 0156	02/12/2007	*Tadarida aegyptiaca*	√		North West	Taung, world heritage site
UP 0157	02/12/2007	*Tadarida aegyptiaca*	√		North West	Taung, world heritage site
UP 0158	02/12/2007	*Tadarida aegyptiaca*	√		North West	Taung, world heritage site
UP 0159	02/12/2007	*Tadarida aegyptiaca*	√		North West	Taung, world heritage site

UP 0160	02/12/2007	*Rhinolophus denti*	√	√	North West	Taung, world heritage site
UP 0161	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0162	02/12/2007	*Rhinolophus denti*		√	North West	Taung, world heritage site
UP 0163	02/12/2007	*Rhinolophus denti*	√	√	North West	Taung, world heritage site
UP 0164	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0165	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0166	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0167	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0168	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0169	02/12/2007	*Neoromicia capensis*	√	√	North West	Taung, world heritage site
UP 0170	02/12/2007	*Eptesicus hottentotus*	√		North West	Taung, world heritage site
UP 0171	02/12/2007	*Rhinolophus denti*	√	√	North West	Taung, world heritage site
UP 0172	02/12/2007	*Rhinolophus denti*	√	√	North West	Taung, world heritage site
UP 0173	02/12/2007	*Eptesicus hottentotus*	√	√	North West	Taung, world heritage site
UP 0174	02/12/2007	*Rhinolophus darlingi*	√	√	North West	Taung, world heritage site
UP 0190	01/12/2007	*Nycteris thebaica*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0191	01/12/2007	*Nycteris thebaica*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0192	01/12/2007	*Rhinolophus simulator*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0193	01/12/2007	*Rhinolophus simulator*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0194	01/12/2007	*Rhinolophus darlingi*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0195	01/12/2007	*Rhinolophus darlingi*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0196	01/12/2007	*Rhinolophus darlingi*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0197	01/12/2007	*Rhinolophus darlingi*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0198	01/12/2007	*Miniopterus natalensis*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0199	01/12/2007	*Miniopterus natalensis*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0200	01/12/2007	*Miniopterus natalensis*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0201	01/12/2007	*Miniopterus natalensis*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0202	01/12/2007	*Miniopterus natalensis*	√	√	North West	Mooiplaats 94 KP, Madikwe Game reserve
UP 0203	01/12/2007	*Scotophilus dinganii*	√	√	North West	Leeuwenhoek 112 KP, Madikwe Game reserve

UP 0204	01/12/2007	*Neoromicia capensis*	√	√	North West	Kalkfontein 111 Kp, Madikwe Game Reserve
UP 0205	01/12/2007	*Sauromys petrophilus*	√	√	North West	Kalkfontein 111 Kp, Madikwe Game Reserve
UP 0206	01/12/2007	*Neoromicia capensis*		√	North West	Kalkfontein 111 Kp, Madikwe Game Reserve
UP 0207	01/12/2007	*Neoromicia capensis*		√	North West	Kalkfontein 111 Kp, Madikwe Game Reserve
UP 0277	2007	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Telkom Umbilo
UP 0284	2007	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Anglican church
UP 0287	07/11/2007	*Miniopterus spp.*		√	North West	Venterskroon Thabela thabeng
UP 0288	07/11/2007	*Miniopterus spp.*		√	North West	Venterskroon Thabela thabeng
UP 0289	07/11/2007	*Miniopterus spp.*		√	North West	Venterskroon Thabela thabeng
UP 0290	07/11/2007	*Miniopterus spp.*		√	North West	Venterskroon Thabela thabeng
UP 0291	07/11/2007	*Miniopterus spp.*		√	North West	Venterskroon Thabela thabeng
UP 0292	07/11/2007	*Miniopterus spp.*		√	North West	Venterskroon Thabela thabeng
UP 0295		*Miniopterus spp.*		√	North West	
UP 0296		*Miniopterus spp.*		√	North West	
UP 0298	14/02/2008	*Epomophorus wahlbergi*	√		Kwa-Zulu Natal	KZN Umkomaas
UP 0299	14/02/2008	*Epomophorus wahlbergi*	√		Kwa-Zulu Natal	KZN Umkomaas
UP 0300	14/02/2008	*Epomophorus wahlbergi*	√		Kwa-Zulu Natal	KZN Umkomaas
UP 0310	24/02/2008	*Neoromicia spp.*		√	Kwa-Zulu Natal	Mpumalanga, Paardeplaas outside Belfast
UP 0312	26/03/2008	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	26 Edinburgh Street, Umkomaas
UP 0313	10/06/2008	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	27 Edinburgh Street, Umkomaas
UP 0314	10/06/2008	*Chaerephon pumilus*		√	Kwa-Zulu	28 Edinburgh Street, Umkomaas

					Natal	
UP 0315	02/04/2008	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Ethewkini, Unicity
UP 0316	27/04/2008	*Epomophorus wahlbergi*		√	Limpopo	Buzzard mountain
UP 0413	2007	*Pipistrellus spp.*		√	Gauteng	Johannesburg
UP 0428	02/12/2008	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	KZN Umkomaas
UP 0469	17/04/2007	*Pipistrellus spp.*		√	Gauteng	Johannesburg
UP 0470	21/01/2008	*Pipistrellus spp.*		√	Gauteng	Johannesburg
UP 0471	2009	*Pipistrellus spp.*		√	Gauteng	Johannesburg
UP 0472	05/12/2008	*Pipistrellus spp.*		√	Gauteng	Henopsriver
UP 0473	10/05/2007	*Mops condylurus*		√	Gauteng	Johannesburg
UP 0474	2007	*Pipistrellus spp.*		√	Gauteng	Johannesburg
UP 0552	27/01/2009	*Pipistrellus spp.*		√	Gauteng	Pretoria
UP 0638	27/09/2010	*Epomophorus spp.*		√	Gauteng	Pretoria Zoo
UP 0639	03/09/2010	*Chiroptera*		√	Gauteng	Henopsriver
UP 0711	01/02/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0712	01/02/2010	*Taphozous mauritianus*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0713	01/02/2010	*Glauconycteris variegatus*		√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0714	01/02/2010	*Glauconycteris variegatus*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0715	01/02/2010	*Glauconycteris variegatus*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0716	01/02/2010	*Scotophilus dinganii*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0717	01/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0718	01/02/2010	*Scotophilus dinganii*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0719	01/02/2010	*Nycticeinops schlieffenii*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0720	01/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0721	01/02/2010	*Nycticeinops schlieffenii*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0722	01/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0723	01/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 1, Pafuri. Kruger National Park

UP 0724	01/02/2010	*Neoromicia nana*		√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0725	01/02/2010	*Neoromicia zuluensis*		√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0726	02/02/2010	*Myotis tricolor*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0727	02/02/2010	*Neoromicia nana*	√	√	Limpopo	Site 1, Pafuri. Kruger National Park
UP 0728	02/02/2010	*Neoromicia nana*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0729	02/02/2010	*Nycticeinops schlieffeni*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0731	02/02/2010	*Rhinolophus fumigatus*	√	√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0732	02/02/2010	*Mops condylurus*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0733	02/02/2010	*Mops condylurus*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0734	02/02/2010	*Mops condylurus*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0735	02/02/2010	*Mops condylurus*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0736	02/02/2010	*Scotophilus viridis*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0737	02/02/2010	*Scotophilus leucogaster*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0738	02/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0739	02/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0740	02/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0741	02/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0742	02/02/2010	*Scotophilus nigrita*	√	√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0743	02/02/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0744	02/02/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0745	02/02/2010	*Pipistrellus rusticus*	√	√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0746	02/02/2010	*Pipistrellus rusticus*	√	√	Limpopo	Site 2, Pafuri. Kruger National Park
UP 0747	02/02/2010	*Pipistrellus rusticus*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0748	02/02/2010	*Pipistrellus rusticus*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0749	02/02/2010	*Pipistrellus rusticus*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0751	02/02/2010	*Scotophilus dinganii*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0755	03/02/2010	*Taphozous mauritianus*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0756	03/02/2010	*Rhinolophus fumigatus*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0757	03/02/2010	*Chaerephon pumilus*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park

UP 0758	03/02/2010	*Chaerephon pumilus*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0759	03/02/2010	*Chaerephon pumilus*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0760	03/02/2010	*Neoromicia nana*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0761	03/02/2010	*Neoromicia nana*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0762	03/02/2010	*Neoromicia helios*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0763	03/02/2010	*Neoromicia helios*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0764	03/02/2010	*Neoromicia helios*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0765	03/02/2010	*Scotophilus dinganii*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0766	03/02/2010	*Scotophilus dinganii*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0767	03/02/2010	*Scotophilus dinganii*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0768	03/02/2010	*Mops condylurus*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0769	03/02/2010	*Taphozous mauritianus*	√		Limpopo	Site 3, Pafuri. Kruger National Park
UP 0769	03/02/2010	*Taphozous mauritianus*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0770	03/02/2010	*Mops condylurus*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0771	03/02/2010	*Mops condylurus*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0772	03/02/2010	*Chaerephon ansorgei*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0773	03/02/2010	*Nycticeinops schlieffenii*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0774	03/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0775	03/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0776	03/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0777	03/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0778	03/02/2010	*Nycticeinops schlieffenii*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0779	03/02/2010	*Scotophilus viridis*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0780	03/02/2010	*Scotophilus leucogaster*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0781	03/02/2010	*Scotophilus viridis*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0782	03/02/2010	*Scotophilus viridis*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0783	03/02/2010	*Scotophilus viridis*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0784	03/02/2010	*Scotophilus viridis*		√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0785	01/02/2010	*Scotophilus leucogaster*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park

UP 0786	01/02/2010	*Scotophilus leucogaster*	√	√	Limpopo	Site 3, Pafuri. Kruger National Park
UP 0787	01/02/2010	*Scotophilus leucogaster*		√	Limpopo	Camp, Pafuri. Kruger National Park
UP 0823	04/02/2010	*Epomophorus wahlbergi*		√	Limpopo	Camp, Pafuri. Kruger National Park
UP 0824	04/02/2010	*Epomophorus wahlbergi*	√	√	Limpopo	Camp, Pafuri. Kruger National Park
UP 0825	04/02/2010	*Epomophorus wahlbergi*	√	√	Limpopo	Camp, Pafuri. Kruger National Park
UP 0825b	12/04/2010	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0826	12/04/2010	*Epomophorus wahlbergi*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0827	12/04/2010	*Glauconycteris variegatus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0828	12/04/2010	*Nycteris thebaica*		√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0829	12/04/2010	*Nycteris thebaica*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0830	12/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0831	12/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0832	12/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0833	12/04/2010	*Scotophilus spp.*		√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0834	12/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0835	12/04/2010	*Chaerephon ansorgei*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0836	12/04/2010	*Chaerephon pumilus*		√	Kwa-Zulu Natal	Rocktail bay: St. Lucia

UP 0837	12/04/2010	*Chaerephon pumilus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0838	12/04/2010	*Chaerephon pumilus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0839	12/04/2010	*Chaerephon pumilus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0840	15/04/2010	*Pipistrellus hesperidus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0841	15/04/2010	*Pipistrellus hesperidus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0842	15/04/2010	*Pipistrellus hesperidus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0843	15/04/2010	*Nycteris thebaica*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0844	15/04/2010	*Kerivoula agentata*		√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0845	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0846	15/04/2010	*Glauconycteris variegatus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0847	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0848	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0849	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0852	15/04/2010	*Scotophilus dinganii*	√		Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0853	15/04/2010	*Epomophorus wahlbergi*		√	Kwa-Zulu	Rocktail bay: St. Lucia

					Natal	
UP 0854	15/04/2010	*Nycteris thebaica*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0855	15/04/2010	*Nycteris thebaica*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0856	15/04/2010	*Nycteris thebaica*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0865	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0866	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0867	15/04/2010	*Scotophilus dinganii*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0868	15/04/2010	*Glauconycteris variegatus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0869	15/04/2010	*Pipistrellus hesperidus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0870	15/04/2010	*Pipistrellus hesperidus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0871	15/04/2010	*Pipistrellus hesperidus*	√	√	Kwa-Zulu Natal	Rocktail bay: St. Lucia
UP 0900	17/05/2009	*Miniopterus spp.*		√	Gauteng	Irene cave
UP 0901	15/01/2010	*Miniopterus spp.*		√	Limpopo	Peppercorn cave, Modimole
UP 0902	18/07/2009	*Miniopterus spp.*		√	Gauteng	Irene cave
UP 0903	18/07/2009	*Miniopterus spp.*		√	Gauteng	Irene cave
UP 0904	18/07/2009	*Miniopterus spp.*		√	Gauteng	Irene cave
UP 0905	13/01/2009	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	23 Star Street, Woodhurst, Chatsworth
UP 0906	28/03/2007	*Epomophorus wahlbergi*		√	Kwa-Zulu	23 Star Street, Woodhurst, Chatsworth

					Natal	
UP 0908	17/07/2008	*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	81 Winfield Drive
UP 0909	08/11/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0910	08/11/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0911	08/11/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0912	08/11/2010	*Hipposideros caffer*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0913	08/11/2010	*Nycticeinops schlieffenii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0914	08/11/2010	*Nycticeinops schlieffenii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0915	08/11/2010	*Neoromicia capensis*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0920	09/11/2010	*Neoromicia nana*		√	Limpopo	Pafuri. Kruger National Park
UP 0921	09/11/2010	*Neoromicia helios*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0922	09/11/2010	*Neoromicia nana*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0923	09/11/2010	*Neoromicia helios*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0927	10/11/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0928	10/11/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0929	10/11/2010	*Scotophilus dinganii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0930	10/11/2010	*Scotophilus dinganii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0931	10/11/2010	*Neoromicia helios*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0932	10/11/2010	*Pipistrellus rusticus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0933	10/11/2010	*Rousettus aegyptiacus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0934	10/11/2010	*Chaerephon pumilus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0937	10/11/2010	*Molossid spp.*	√		Limpopo	Pafuri. Kruger National Park
UP 0947	11/11/2010	*Rhinolophus landeri*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0948	11/11/2010	*Hipposideros caffer*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0949	12/11/2010	*Hipposideros caffer*		√	Limpopo	Pafuri. Kruger National Park
UP 0950	12/11/2010	*Epomophorus wahlbergi*		√	Limpopo	Pafuri. Kruger National Park
UP 0951	12/11/2010	*Hipposideros caffer*		√	Limpopo	Pafuri. Kruger National Park
UP 0952	12/11/2010	*Neoromicia helios*	√	√	Limpopo	Pafuri. Kruger National Park

UP 0953	12/11/2010	*Taphozous mauritianus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0954	12/11/2010	*Scotophilus viridis*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0955	12/11/2010	*Scotophilus dinganii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0956	12/11/2010	*Scotophilus dinganii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0957	12/11/2010	*Scatphalous spp.*		√	Limpopo	Pafuri. Kruger National Park
UP 0958	12/11/2010	*Scotophilus dinganii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0959	12/11/2010	*Scotophilus dinganii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0960	12/11/2010	*Scotophilus leucogaster*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0961	12/11/2010	*Neoromicia zuluensis*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0962	12/11/2010	*Neoromicia nana*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0963	12/11/2010	*Neoromicia capensis*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0964	12/11/2010	*Neoromicia rueppellii*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0965	12/11/2010	*Chaerephon ansorgei*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0966	12/11/2010	*Mops condylurus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0967	12/11/2010	*Mops condylurus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 0968	12/11/2010	*Mops condylurus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 1003	12/11/2010	*Epomophorus wahlbergi*	√	√	Limpopo	Pafuri. Kruger National Park
UP 1004	12/11/2010	*Epomophorus gambianus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 1005	12/11/2010	*Epomophorus gambianus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 1006	12/11/2010	*Epomophorus gambianus*	√	√	Limpopo	Pafuri. Kruger National Park
UP 1007	11/12/2010	*Tadarida aegyptiaca*	√	√	North West	Kgaswane nature reserve
UP 1008	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1009	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1010	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1011	11/12/2010	*Pipistrellus rusticus*		√	North West	Kgaswane nature reserve
UP 1012	11/12/2010	*Neoromicia capensis*	√	√	North West	Kgaswane nature reserve
UP 1013	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1014	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1015	11/12/2010	*Scotophilus dinganii*		√	North West	Kgaswane nature reserve

UP 1016	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1017	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1018	11/12/2010	*Scotophilus spp.*	√	√	North West	Kgaswane nature reserve
UP 1019	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1020	11/12/2010	*Scotophilus dinganii*	√	√	North West	Kgaswane nature reserve
UP 1021	11/12/2010	*Neoromicia capensis*	√	√	North West	Kgaswane nature reserve
UP 1022	11/12/2010	*microbat*	√	√	North West	Kgaswane nature reserve
UP 1025	11/12/2010	*Scotophilus spp.*		√	North West	Kgaswane nature reserve
UP 1052	11/12/2010	*Neoromicia capensis*		√	North West	Kgaswane nature reserve
UP 1053		*Epomophorus wahlbergi*		√	Kwa-Zulu Natal	23 Star Street, Woodhurst, Chatsworth
UP 1054	03/12/2010	*Neoromicia capensis*		√	Gauteng	
UP 1055		*Chiroptera*		√	Gauteng	
UP 1056	01/12/2010	*Chiroptera*		√	Gauteng	
UP 1361	28/02/2006	*Neoromicia capensis*		√	Gauteng	
UP 1362	17/03/2006	*Neoromicia capensis*		√	Gauteng	
UP 1363	13/09/2011	*Scotophilus dinganii*		√	Limpopo	71 Kameel St. Louis Trichardt
UP 1364	13/09/2011	*Mops midas*		√	Limpopo	71 Kameel St. Louis Trichardt
UP 1365	13/09/2011	*Epomophorus wahlbergi*		√	Limpopo	Buzzard Mountain, Louis Trichardt
UP 1366	14/09/2011	*Rhinolophus simulator*		√	Limpopo	Labuschagne farm, Louis Trichardt
UP 1367	14/09/2011	*Rhinolophus capensis*		√	Limpopo	Labuschagne farm, Louis Trichardt
UP 1368	14/09/2011	*Rhinolophus hildebrandtii*		√	Limpopo	Labuschagne farm, Louis Trichardt
UP 1369	24/09/2011	*Neoromicia capensis*		√	Gauteng	Free Me rehab
UP 1373	01/10/2010	*Rhinolophus spp.*		√	Gauteng	Irene cave - ARC
UP 1374	01/04/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1375	01/04/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1376	01/04/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1377	01/04/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1378	01/04/2011	*Miniopterus natalensis*		√	Gauteng	Irene caves

UP 1380	01/04/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1381	01/04/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1382	06/05/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1382	06/05/2011	*Miniopterus natalensis*		√	Gauteng	Irene caves
UP 1383	06/05/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1384	06/05/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1385	06/05/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1386	06/05/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1387	06/05/2011	*Miniopterus natalensis*		√	Gauteng	Irene caves
UP 1388	03/06/2011	*Miniopterus natalensis*		√	Gauteng	Irene caves
UP 1389	03/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1390	03/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1391	03/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1392	03/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1393	03/06/2011	*Miniopterus natalensis*		√	Gauteng	Irene caves
UP 1394	03/06/2011	*Miniopterus natalensis*		√	Gauteng	Irene caves
UP 1395	29/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1396	29/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1397	29/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1398	29/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1399	29/06/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1400	25/07/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1401	25/07/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1402	25/07/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1403	25/07/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1404	25/07/2011	*Miniopterus natalensis*	√	√	Gauteng	Irene caves
UP 1405	06/10/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1406	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1407	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves

UP 1408	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1409	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1410	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1411	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1413	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1414	01/07/2011	*Miniopterus natalensis*	√		Gauteng	Irene caves
UP 1421	27/11/2011	*Miniopterus natalensis*		√	Gauteng	
UP 1422	05/12/2011	*Miniopterus natalensis*		√	Gauteng	
UP 1423	17/12/2011	*Miniopterus natalensis*		√	Gauteng	
UP 1424		*Miniopterus natalensis*		√	Gauteng	
UP 1425		*Miniopterus natalensis*		√	Gauteng	
UP 1426		*Miniopterus natalensis*		√	Gauteng	
UP 1427		*Miniopterus natalensis*		√	Gauteng	
UP 1443	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1456	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1465	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1471	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1482	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1483	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1484	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1485	12/06/2012	*Rhinolophus spp.*		√	Limpopo	Matlapitsi cave
UP 1486	12/06/2012	*Rhinolophus spp.*		√	Limpopo	Matlapitsi cave
UP 1487	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1488	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1489	12/06/2012	*Rhinolophus spp.*		√	Limpopo	Matlapitsi cave
UP 1490	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1491	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1492	12/06/2012	*Rousettus aegyptiacus*	√	√	Limpopo	Matlapitsi cave
UP 1493	12/06/2012	*Rhinolophus spp.*		√	Limpopo	Matlapitsi cave

UP 1494	12/06/2012	*Rhinolophus spp.*		√	Limpopo	Matlapitsi cave
UP 1495	12/06/2012	*Rhinolophus spp.*		√	Limpopo	Matlapitsi cave
UP 1496	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1497	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1498	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1499	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1500	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1501	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1502	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1503	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1504	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1505	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1506	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1507	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1508	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1509	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1510	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1511	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1512	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1513	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1514	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1515	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1516	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1517	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1518	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1519	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1520	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1521	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1522	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave

UP 1523	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1524	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1525	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1526	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1527	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1528	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1529	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1530	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1531	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1532	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1533	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1534	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1535	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1536	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1537	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1538	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1539	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1540	19/08/2012	*Nycteris thebaica*		√	Limpopo	Rooiberg (Monate)
UP 1541	19/08/2012	*Nycteris thebaica*		√	Limpopo	Rooiberg (Monate)
UP 1542	19/08/2012	*Nycteris thebaica*		√	Limpopo	Rooiberg (Monate)
UP 1543	19/08/2012	*Nycteris thebaica*		√	Limpopo	Rooiberg (Monate)
UP 1544	19/08/2012	*Rhinolophus spp.*		√	Limpopo	Rooiberg (Monate)
UP 1545	19/08/2012	*Rhinolophus spp.*		√	Limpopo	Rooiberg (Monate)
UP 1546	19/08/2012	*Rhinolophus spp.*		√	Limpopo	Rooiberg (Monate)
UP 1547		*Chaerephon pumilus*		√	Gauteng	Pretoria Zoo
UP 1614	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1615	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1616	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1668	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave

UP 1617	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1618	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1619	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1620	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1621	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1622	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1623	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1624	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1625	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1626	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1627	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1628	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1629	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1630	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1631	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1632	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1633	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1634	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1635	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1636	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1637	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1638	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1639	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1640	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1641	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1642	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1643	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1644	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1645	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave

UP 1646	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1647	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1648	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1649	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1650	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1651	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1652	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1653	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1654	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1655	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1656	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1657	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1658	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1659	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1660	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1661	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1662	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1663	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1664	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1665	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1666	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1667	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1669	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1670	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1671	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1672	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1673	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1674	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1675	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave

UP 1676	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1677	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1678	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1679	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1680	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1681	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1682	27/06/2012	*Rousettus aegyptiacus*	√		Limpopo	Matlapitsi cave
UP 1700	16/01/2013	*Rhinolophus simulator*		√	North West	Donkerpoort, Gatkop
UP 1701	16/01/2013	*Rhinolophus simulator*	√	√	North West	Donkerpoort, Gatkop
UP 1702	16/01/2013	*Miniopterus natalensis*	√	√	North West	Donkerpoort, Gatkop
UP 1703	17/01/2013	*Miniopterus natalensis*	√	√	North West	Donkerpoort, Gatkop
UP 1704	16/01/2013	*Miniopterus natalensis*	√	√	North West	Donkerpoort, Gatkop
UP 1705	16/01/2013	*Miniopterus natalensis*		√	North West	Donkerpoort, Gatkop
UP 1706	16/01/2013	*Miniopterus natalensis*		√	North West	Donkerpoort, Gatkop
UP 1717	18/01/2013	*Miniopterus natalensis*	√	√	North West	River, Gatkop
UP 1718	18/01/2013	*Miniopterus natalensis*	√	√	North West	River, Gatkop
UP 1719	18/01/2013	*Miniopterus natalensis*	√	√	North West	River, Gatkop
UP 1723	18/01/2013	*Miniopterus natalensis*		√	North West	River, Gatkop
UP 1731	18/01/2013	*Miniopterus natalensis*	√	√	North West	River, Gatkop
UP 1755	18/01/2013	*Miniopterus natalensis*		√	North West	River, Gatkop
UP 1764	20/01/2013	*Miniopterus natalensis*	√		North West	Homestead, Gatkop
UP 1786	20/01/2013	*Miniopterus natalensis*		√	North West	Homestead, Gatkop
UP 1787	20/01/2013	*Neoromicia zuluensis*	√	√	North West	Homestead, Gatkop
UP 1788	20/01/2013	*Pipistrellus rusticus*	√	√	North West	Homestead, Gatkop
UP 1789	20/01/2013	*Rhinolophus simulator*	√		North West	Homestead, Gatkop
UP 1790	20/01/2013	*Rhinolophus simulator*	√	√	North West	Homestead, Gatkop
UP 1791	20/01/2013	*Rhinolophus simulator*	√	√	North West	Homestead, Gatkop
UP 1792	20/01/2013	*Scotophilus spp.*	√	√	North West	Homestead, Gatkop
UP 1793	20/01/2013	*Scotophilus spp.*	√	√	North West	Homestead, Gatkop

UP 1794	20/01/2013	*Scotophilus spp.*	√	√	North West	Homestead, Gatkop
UP 1795	20/01/2013	*Scotophilus spp.*	√	√	North West	Homestead, Gatkop
UP 1801	21/01/2013	*Miniopterus natalensis*		√	North West	Site A, Gatkop
UP 1807	22/01/2013	*Nycteris thebaica*	√		North West	Gatkop cave
UP 1808	22/01/2013	*Nycteris thebaica*	√	√	North West	Gatkop cave
UP 1809	22/01/2013	*Nycteris thebaica*	√		North West	Gatkop cave
UP 1810	22/01/2013	*Nycteris thebaica*	√	√	North West	Gatkop cave
UP 1811	22/01/2013	*Nycteris thebaica*	√	√	North West	Gatkop cave
UP 1812	22/01/2013	*Nycteris thebaica*	√		North West	Gatkop cave

Appendix S2. Morphologically identified bats collected in South Africa that were used for the construction of COI DNA barcoding sequences.

UP number	Province	Description of locality	Species name	NCBI
UP 0759	Limpopo	Pafuri. Kruger National Park	*Chaerephon pumilus*	KF452602
UP 0835	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Chaerephon pumilus*	KF452603
UP 0838	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Chaerephon pumilus*	KF452604
UP 0839	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Chaerephon pumilus*	KF452605
UP 1004	Limpopo	Pafuri. Kruger National Park	*Epomophorus gambianus*	KF452606
UP 1005	Limpopo	Pafuri. Kruger National Park	*Epomophorus gambianus*	KF452607
UP 0306	Kwa-Zulu Natal	Durban	*Epomophorus gambianus*	KF452608
UP 0307	Kwa-Zulu Natal	Durban	*Epomophorus gambianus*	KF452609
UP 0309	Kwa-Zulu Natal	Durban	*Epomophorus gambianus*	KF452610
UP 0301	Kwa-Zulu Natal	Durban	*Epomophorus wahlbergi*	KF452611
UP 0305	Kwa-Zulu Natal	Durban	*Epomophorus wahlbergi*	KF452612
UP 0823	Limpopo	Pafuri. Kruger National Park	*Epomophorus wahlbergi*	KF452613
UP 0824	Limpopo	Pafuri. Kruger National Park	*Epomophorus wahlbergi*	KF452614
UP 0825	Limpopo	Pafuri. Kruger National Park	*Epomophorus wahlbergi*	KF452615
UP 0905	Kwa-Zulu Natal	23 Star Street, Woodhurst, Chatsworth	*Epomophorus wahlbergi*	KF452616
UP 0906	Kwa-Zulu Natal	23 Star Street, Woodhurst, Chatsworth	*Epomophorus wahlbergi*	KF452617
UP 0908	Kwa-Zulu Natal	81 Winfield Drive	*Epomophorus wahlbergi*	KF452618
UP 0950	Limpopo	Pafuri. Kruger National Park	*Epomophorus wahlbergi*	KF452619
UP 1003	Limpopo	Pafuri. Kruger National Park	*Epomophorus wahlbergi*	KF452620
UP 0170	North West	Taung	*Eptesicus hottentotus*	KF452621
UP 0173	North West	Taung	*Eptesicus hottentotus*	KF452622
UP 0912	Limpopo	Pafuri. Kruger National Park	*Hipposideros caffer*	KF452623
UP 0949	Limpopo	Pafuri. Kruger National Park	*Hipposideros caffer*	KF452624
UP 0198	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Miniopterus natalensis*	KF452625
UP 0201	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Miniopterus natalensis*	KF452626
UP 0733	Limpopo	Pafuri. Kruger National Park	*Mops condylurus*	KF452627
UP 0768	Limpopo	Pafuri. Kruger National Park	*Mops condylurus*	KF452628
UP 0770	Limpopo	Pafuri. Kruger National Park	*Mops condylurus*	KF452629

UP 0771	Limpopo	Pafuri. Kruger National Park	*Mops condylurus*	KF452630
UP 0966	Limpopo	Pafuri. Kruger National Park	*Mops condylurus*	KF452631
UP 0161	North West	Taung	*Neoromicia capensis*	KF452632
UP 0164	North West	Taung	*Neoromicia capensis*	KF452633
UP 0165	North West	Taung	*Neoromicia capensis*	KF452634
UP 0166	North West	Taung	*Neoromicia capensis*	KF452635
UP 0167	North West	Taung	*Neoromicia capensis*	KF452636
UP 0168	North West	Taung	*Neoromicia capensis*	KF452637
UP 0169	North West	Taung	*Neoromicia capensis*	KF452638
UP 0204	North West	Kalkfontein 111 Kp, Madikwe Game Reserve	*Neoromicia capensis*	KF452639
UP 0206	North West	Kalkfontein 111 Kp, Madikwe Game Reserve	*Neoromicia capensis*	KF452640
UP 0207	North West	Kalkfontein 111 Kp, Madikwe Game Reserve	*Neoromicia capensis*	KF452641
UP 0915	Limpopo	Pafuri. Kruger National Park	*Neoromicia capensis*	KF452642
UP 0963	Limpopo	Pafuri. Kruger National Park	*Neoromicia capensis*	KF452643
UP 0921	Limpopo	Pafuri. Kruger National Park	*Neoromicia helios*	KF452644
UP 0923	Limpopo	Pafuri. Kruger National Park	*Neoromicia nana*	KF452645
UP 0931	Limpopo	Pafuri. Kruger National Park	*Neoromicia nana*	KF452646
UP 0922	Limpopo	Pafuri. Kruger National Park	*Neoromicia nana*	KF452647
UP 0964	Limpopo	Pafuri. Kruger National Park	*Neoromicia rueppellii*	KF452648
UP 0725	Limpopo	Pafuri. Kruger National Park	*Neoromicia zuluensis*	KF452649
UP 0190	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Nycteris thebaica*	KF452650
UP 0191	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Nycteris thebaica*	KF452651
UP 0829	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Nycteris thebaica*	KF452652
UP 0843	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Nycteris thebaica*	KF452653
UP 0856	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Nycteris thebaica*	KF452654
UP 0719	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452655
UP 0720	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452656
UP 0773	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452657
UP 0775	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452658
UP 0776	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452659

UP 0777	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452660
UP 0778	Limpopo	Pafuri. Kruger National Park	*Nycticeinops schlieffenii*	KF452661
UP 0111	Kwa-Zulu Natal	Durban	*Otomops martiensseni*	KF452662
UP 0840	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Pipistrellus hesperidus*	KF452663
UP 0841	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Pipistrellus hesperidus*	KF452664
UP 0842	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Pipistrellus hesperidus*	KF452665
UP 0871	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Pipistrellus hesperidus*	KF452666
UP 0745	Limpopo	Pafuri. Kruger National Park	*Pipistrellus rusticus*	KF452667
UP 0746	Limpopo	Pafuri. Kruger National Park	*Pipistrellus rusticus*	KF452668
UP 0747	Limpopo	Pafuri. Kruger National Park	*Pipistrellus rusticus*	KF452669
UP 0748	Limpopo	Pafuri. Kruger National Park	*Pipistrellus rusticus*	KF452670
UP 0749	Limpopo	Pafuri. Kruger National Park	*Pipistrellus rusticus*	KF452671
UP 0932	Limpopo	Pafuri. Kruger National Park	*Pipistrellus rusticus*	KF452672
UP 0195	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Rhinolophus darlingi*	KF452673
UP 0196	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Rhinolophus darlingi*	KF452674
UP 0197	North West	Mooiplaats 94 KP, Madikwe Game reserve	*Rhinolophus darlingi*	KF452675
UP 0162	North West	Taung	*Rhinolophus denti*	KF452676
UP 0171	North West	Taung	*Rhinolophus denti*	KF452677
UP 0172	North West	Taung	*Rhinolophus denti*	KF452678
UP 0711	Limpopo	Pafuri. Kruger National Park	*Rousettus aegyptiacus*	KF452679
UP 0909	Limpopo	Pafuri. Kruger National Park	*Rousettus aegyptiacus*	KF452680
UP 0911	Limpopo	Pafuri. Kruger National Park	*Rousettus aegyptiacus*	KF452681
UP 0928	Limpopo	Pafuri. Kruger National Park	*Rousettus aegyptiacus*	KF452682
UP 0933	Limpopo	Pafuri. Kruger National Park	*Rousettus aegyptiacus*	KF452683
UP 0205	North West	Kalkfontein 111 Kp, Madikwe Game Reserve	*Sauromys petrophilus*	KF452684
UP 0203	North West	Leeuwenhoek 112 KP, Madikwe Game reserve	*Scotophilus dinganii*	KF452685
UP 0716	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452686
UP 0718	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452687
UP 0722	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452688
UP 0723	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452689

UP 0767	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452690
UP 0831	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452691
UP 0847	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Scotophilus dinganii*	KF452692
UP 0849	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Scotophilus dinganii*	KF452693
UP 0867	Kwa-Zulu Natal	Rocktail bay: St. Lucia	*Scotophilus dinganii*	KF452694
UP 0929	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452695
UP 0930	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452696
UP 0956	Limpopo	Pafuri. Kruger National Park	*Scotophilus dinganii*	KF452697
UP 0737	Limpopo	Pafuri. Kruger National Park	*Scotophilus leucogaster*	KF452698
UP 0780	Limpopo	Pafuri. Kruger National Park	*Scotophilus leucogaster*	KF452699
UP 0785	Limpopo	Pafuri. Kruger National Park	*Scotophilus leucogaster*	KF452700
UP 0786	Limpopo	Pafuri. Kruger National Park	*Scotophilus leucogaster*	KF452701
UP 0787	Limpopo	Pafuri. Kruger National Park	*Scotophilus leucogaster*	KF452702
UP 0960	Limpopo	Pafuri. Kruger National Park	*Scotophilus leucogaster*	KF452703
UP 0742	Limpopo	Pafuri. Kruger National Park	*Scotophilus nigrita*	KF452704
UP 0736	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452705
UP 0779	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452706
UP 0781	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452707
UP 0782	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452708
UP 0783	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452709
UP 0784	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452710
UP 0954	Limpopo	Pafuri. Kruger National Park	*Scotophilus viridis*	KF452711
UP 0156	North West	Taung	*Tadarida aegyptiaca*	KF452712
UP 0157	North West	Taung	*Tadarida aegyptiaca*	KF452713
UP 0158	North West	Taung	*Tadarida aegyptiaca*	KF452714
UP 0159	North West	Taung	*Tadarida aegyptiaca*	KF452715
UP 0712	Limpopo	Pafuri. Kruger National Park	*Taphozous mauritianus*	KF452716
UP 0769	Limpopo	Pafuri. Kruger National Park	*Taphozous mauritianus*	KF452717
UP 0953	Limpopo	Pafuri. Kruger National Park	*Taphozous mauritianus*	KF452718

Appendix S3. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the 17 *Epomophorus spp*. samples from South Africa.

	UP_301	UP_305	UP_306	UP_307	UP_309	UP_823	UP_824	UP_825	UP_905	UP_906	UP_908	UP_950	UP_1003	UP_1004	UP_1005	AAC2947
UP_301_Epomophorus_wahlbergi																
UP_305_Epomophorus_wahlbergi	0.057															
UP_306_Epomophorus_gambianus	0.059	0.006														
UP_307_Epomophorus_gambianus	0.055	0.002	0.004													
UP_309_Epomophorus_gambianus	0.057	0.009	0.011	0.008												
UP_823_Epomophorus_wahlbergi	0.002	0.055	0.057	0.053	0.055											
UP_824_Epomophorus_wahlbergi	0.057	0.008	0.009	0.006	0.013	0.055										
UP_825_Epomophorus_wahlbergi	0.057	0.008	0.009	0.006	0.013	0.055	0.011									
UP_905_Epomophorus_wahlbergi	0.002	0.055	0.057	0.053	0.055	0.000	0.055	0.055								
UP_906_Epomophorus_wahlbergi	0.059	0.006	0.008	0.004	0.011	0.057	0.009	0.009	0.057							
UP_908_Epomophorus_wahlbergi	0.008	0.059	0.061	0.057	0.059	0.006	0.059	0.059	0.006	0.062						
UP_950_Epomophorus_wahlbergi	0.002	0.055	0.057	0.053	0.055	0.000	0.055	0.055	0.000	0.057	0.006					
UP_1003_Epomophorus_wahlbergi	0.011	0.057	0.059	0.055	0.057	0.009	0.057	0.057	0.009	0.059	0.015	0.009				
UP_1004_Epomophorus_gambianus	0.057	0.004	0.002	0.002	0.009	0.055	0.008	0.008	0.055	0.006	0.059	0.055	0.057			
UP_1005_Epomophorus_gambianus	0.053	0.008	0.006	0.006	0.013	0.051	0.011	0.011	0.051	0.009	0.055	0.051	0.053	0.004		
Epomophorus gambianus (_BOLD:AAC2947)	0.059	0.021	0.023	0.019	0.023	0.057	0.025	0.025	0.057	0.023	0.062	0.057	0.059	0.021	0.021	
Epomophorus wahlbergi (GenBank:_JF442398.1)	0.011	0.053	0.055	0.051	0.053	0.009	0.053	0.053	0.009	0.055	0.015	0.009	0.011	0.053	0.049	0.055

Appendix S4. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the five Rousettus aegyptiacus samples from South Africa.

	UP_933	UP_711	UP_909	UP_911	UP_928	AAA2863
UP_933_Rousettus_aegyptiacus						
UP_711_Rousettus_aegyptiacus	0.025					
UP_909_Rousettus_aegyptiacus	0.023	0.018				
UP_911_Rousettus_aegyptiacus	0.002	0.027	0.025			
UP_928_Rousettus_aegyptiacus	0.002	0.027	0.025	0.003		
Rousettus aegyptiacus_(BOLD:AAA2863)	0.022	0.013	0.015	0.023	0.023	
Rousettus_aegyptiacus_JF444434	0.022	0.013	0.015	0.023	0.023	0.000

Appendix S5. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the five Nycteris spp. samples from South Africa.

	UP_190	UP_191	UP_829	UP_843	UP_856	AAI3441
UP_190_Nycteris_thebaica						
UP_191_Nycteris_thebaica	0.128					
UP_829_Nycteris_thebaica	0.208	0.172				
UP_843_Nycteris_thebaica	0.208	0.172	0.000			
UP_856_Nycteris_thebaica	0.208	0.172	0.000	0.000		
Nycteris grandis_(BOLD:AAI3441)	0.219	0.211	0.237	0.237	0.237	
Nycteris thebaica_(GenBank:_JF442546.1)	0.220	0.183	0.047	0.047	0.047	0.232

Appendix S6. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the 27 Scotophilus spp. samples from South Africa.

	UP_203	UP_716	UP_718	UP_722	UP_723	UP_736	UP_737	UP_742	UP_767	UP_779	UP_780	UP_781	UP_782	UP_783	UP_784	UP_785	UP_786	UP_787	UP_831	UP_847	UP_849	UP_867	UP_929	UP_930	UP_954	UP_956	UP_960	JF442688.1	AAD3569	AAC0094
UP_203_Scotophilus_dinganii																														
UP_716_Scotophilus_dinganii	0.011																													
UP_718_Scotophilus_dinganii	0.011	0.000																												
UP_722_Scotophilus_dinganii	0.011	0.000	0.000																											
UP_723_Scotophilus_dinganii	0.011	0.000	0.000	0.000																										
UP_736_Scotophilus_viridis	0.140	0.131	0.131	0.131	0.131																									
UP_737_Scotophilus_leucogaster	0.140	0.131	0.131	0.131	0.131	0.000																								
UP_742_Scotophilus_nigrita	0.120	0.134	0.134	0.134	0.134	0.114	0.114																							
UP_767_Scotophilus_dinganii	0.014	0.002	0.002	0.002	0.002	0.129	0.129	0.137																						
UP_779_Scotophilus_viridis	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129																					
UP_780_Scotophilus_leucogaster	0.143	0.134	0.134	0.134	0.134	0.002	0.002	0.111	0.131	0.002																				

Continued	UP_203	UP_716	UP_718	UP_722	UP_723	UP_736	UP_737	UP_742	UP_767	UP_779	UP_780	UP_781	UP_782	UP_783	UP_784	UP_785	UP_786	UP_787	UP_831	UP_847	UP_849	UP_867	UP_929	UP_930	UP_954	UP_956	UP_960	JF442688.1	AAD3569	AAC0094
UP_781_Scotophilus_viridis	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002																			
UP_782_Scotophilus_viridis	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002	0.000																		
UP_783_Scotophilus_viridis	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002	0.000	0.000																	
UP_784_Scotophilus_viridis	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002	0.000	0.000	0.000																
UP_785_Scotophilus_leucogaster	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002	0.000	0.000	0.000	0.000															
UP_786_Scotophilus_leucogaster	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002	0.000	0.000	0.000	0.000	0.000														
UP_787_Scotophilus_leucogaster	0.140	0.131	0.131	0.131	0.131	0.000	0.000	0.114	0.129	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000													
UP_831_Scotophilus_dinganii	0.016	0.014	0.014	0.014	0.014	0.145	0.145	0.136	0.016	0.145	0.148	0.145	0.145	0.145	0.145	0.145	0.145	0.145												
UP_847_Scotophilus_dinganii	0.011	0.009	0.009	0.009	0.009	0.140	0.140	0.131	0.011	0.140	0.142	0.140	0.140	0.140	0.140	0.140	0.140	0.140	0.004											
UP_849_Scotophilus_dinganii	0.016	0.014	0.014	0.014	0.014	0.145	0.145	0.136	0.016	0.145	0.148	0.145	0.145	0.145	0.145	0.145	0.145	0.145	0.000	0.004										
UP_867_Scotophilus_dinganii	0.014	0.011	0.011	0.011	0.011	0.137	0.137	0.133	0.009	0.137	0.140	0.137	0.137	0.137	0.137	0.137	0.137	0.137	0.007	0.002	0.007									
UP_929_Scotophilus_dinganii	0.011	0.000	0.000	0.000	0.000	0.131	0.131	0.134	0.002	0.131	0.134	0.131	0.131	0.131	0.131	0.131	0.131	0.131	0.014	0.009	0.014	0.011								

Continued 2	UP_203	UP_716	UP_718	UP_722	UP_723	UP_736	UP_737	UP_742	UP_767	UP_779	UP_780	UP_781	UP_782	UP_783	UP_784	UP_785	UP_786	UP_787	UP_831	UP_847	UP_849	UP_867	UP_929	UP_930	UP_954	UP_956	UP_960	JF442688.1	AAD3569	AAC0094
UP_930_Scotophilus_ dinganii	0.011	0.000	0.000	0.000	0.000	0.131	0.131	0.134	0.002	0.131	0.134	0.131	0.131	0.131	0.131	0.131	0.131	0.131	0.014	0.009	0.014	0.011	0.000							
UP_954_Scotophilus_ viridis	0.143	0.134	0.134	0.134	0.134	0.002	0.002	0.111	0.131	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.148	0.142	0.148	0.140	0.134	0.134						
UP_956_Scotophilus_ dinganii	0.011	0.000	0.000	0.000	0.000	0.131	0.131	0.134	0.002	0.131	0.134	0.131	0.131	0.131	0.131	0.131	0.131	0.131	0.014	0.009	0.014	0.011	0.000	0.000	0.134					
UP_960_Scotophilus_ leucogaster	0.143	0.134	0.134	0.134	0.134	0.002	0.002	0.111	0.131	0.002	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.148	0.142	0.148	0.140	0.134	0.134	0.000	0.134				
Scotophilus dinganii (GenBank:_JF442688.1)	0.115	0.112	0.112	0.112	0.112	0.120	0.120	0.129	0.109	0.120	0.123	0.120	0.120	0.120	0.120	0.120	0.120	0.120	0.120	0.120	0.120	0.117	0.112	0.112	0.123	0.112	0.123			
Scotophilus heathi (BOLD:AAD3569)	0.151	0.145	0.145	0.145	0.145	0.157	0.157	0.147	0.142	0.157	0.160	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.153	0.153	0.153	0.150	0.145	0.145	0.160	0.145	0.160	0.137		
Scotophilus kuhli (BOLD:AAC0094)	0.230	0.237	0.237	0.237	0.237	0.194	0.194	0.181	0.234	0.194	0.191	0.194	0.194	0.194	0.194	0.194	0.194	0.194	0.236	0.236	0.236	0.233	0.237	0.237	0.191	0.237	0.191	0.194	0.157	
Scotophilus kuhli HM541937	0.230	0.237	0.237	0.237	0.237	0.201	0.201	0.175	0.241	0.201	0.198	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.236	0.236	0.236	0.240	0.237	0.237	0.198	0.237	0.198	0.201	0.163	0.004

Appendix S7. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the 18 Neoromicia spp. samples from South Africa.

	UP_161	UP_164	UP_165	UP_166	UP_167	UP_168	UP_169	UP_204	UP_206	UP_207	UP_725	UP_915	UP_921	UP_922	UP_923	UP_931	UP_963	UP_964	JF444136.1
UP_161_Neoromicia_capensis																			
UP_164_Neoromicia_capensis	0.000																		
UP_165_Neoromicia_capensis	0.000	0.000																	
UP_166_Neoromicia_capensis	0.002	0.002	0.002																
UP_167_Neoromicia_capensis	0.002	0.002	0.002	0.000															
UP_168_Neoromicia_capensis	0.000	0.000	0.000	0.002	0.002														
UP_169_Neoromicia_capensis	0.002	0.002	0.002	0.000	0.000	0.002													
UP_204_Neoromicia_capensis	0.000	0.000	0.000	0.002	0.002	0.000	0.002												
UP_206_Neoromicia_capensis	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.000											
UP_207_Neoromicia_capensis	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.000	0.000										
UP_725_Neoromicia_zuluensis	0.157	0.157	0.157	0.154	0.154	0.157	0.154	0.157	0.157	0.157									
UP_915_Neoromicia_capensis	0.004	0.004	0.004	0.002	0.002	0.004	0.002	0.004	0.004	0.004	0.154								
UP_921_Neuromicia_helios	0.153	0.153	0.153	0.150	0.150	0.153	0.150	0.153	0.153	0.153	0.185	0.150							
UP_922_Neuromicia_nana	0.147	0.147	0.147	0.145	0.145	0.147	0.145	0.147	0.147	0.147	0.194	0.145	0.033						
UP_923_Neoromicia_nana	0.158	0.158	0.158	0.155	0.155	0.158	0.155	0.158	0.158	0.158	0.202	0.155	0.049	0.020					
UP_931_Neoromicia_nana	0.145	0.145	0.145	0.142	0.142	0.145	0.142	0.145	0.145	0.145	0.188	0.142	0.035	0.006	0.013				

Continued	UP_161	UP_164	UP_165	UP_166	UP_167	UP_168	UP_169	UP_204	UP_206	UP_207	UP_725	UP_915	UP_921	UP_922	UP_923	UP_931	UP_963	UP_964	JF444136.1
UP_963_Neoromicia_capensis	0.007	0.007	0.007	0.004	0.004	0.007	0.004	0.007	0.007	0.007	0.154	0.007	0.156	0.150	0.161	0.148			
UP_964_Neoromicia_rueppellii	0.007	0.007	0.007	0.004	0.004	0.007	0.004	0.007	0.007	0.007	0.154	0.007	0.156	0.150	0.161	0.148	0.000		
Neoromicia brunneus (GenBank:_JF444136.1)	0.170	0.170	0.170	0.167	0.167	0.170	0.167	0.170	0.170	0.170	0.195	0.167	0.173	0.173	0.178	0.170	0.170	0.170	
Neoromicia nanus (GenBank:_JF444202.1)	0.145	0.145	0.145	0.142	0.142	0.145	0.142	0.145	0.145	0.145	0.192	0.142	0.031	0.011	0.022	0.009	0.148	0.148	0.165

Appendix S8. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the 10 Pipistrellus spp. samples from South Africa.

	UP_745	UP_746	UP_747	UP_748	UP_749	UP_840	UP_841	UP_842	UP_871	UP_932	AAH9463
UP_745_Pipistrellus_rusticus											
UP_746_Pipistrellus_rusticus	0.004										
UP_747_Pipistrellus_rusticus	0.006	0.002									
UP_748_Pipistrellus_rusticus	0.004	0.000	0.002								
UP_749_Pipistrellus_rusticus	0.006	0.002	0.004	0.002							
UP_840_Pipistrellus_hesperidus	0.153	0.153	0.151	0.153	0.156						
UP_841_Pipistrellus_hesperidus	0.146	0.146	0.144	0.146	0.149	0.019					
UP_842_Pipistrellus_hesperidus	0.153	0.153	0.151	0.153	0.156	0.000	0.019				
UP_871_Pipistrellus_hesperidus	0.153	0.153	0.151	0.153	0.156	0.000	0.019	0.000			
UP_932_Pipistrellus_rusticus	0.006	0.002	0.004	0.002	0.004	0.151	0.144	0.151	0.151		
Pipistrellus eisentrauti (BOLD:AAH9463)	0.217	0.217	0.214	0.217	0.220	0.217	0.217	0.217	0.217	0.220	
Pipistrellus tenuis_(BOLD:AAB2554)	0.201	0.207	0.204	0.207	0.207	0.199	0.204	0.199	0.199	0.204	0.226

Appendix S9. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the six Rhinolophus spp. samples from South Africa.

	UP_172	UP_162	UP_171	UP_195	UP_196	UP_197	AAD6851	AAD0389
UP_172_Rhinolophus_denti								
UP_162_Rhinolophus_denti	0.004							
UP_171_Rhinolophus_denti	0.000	0.004						
UP_195_Rhinolophus_darlingi	0.166	0.161	0.166					
UP_196_Rhinolophus_darlingi	0.163	0.158	0.163	0.002				
UP_197_Rhinolophus_darlingi	0.163	0.158	0.163	0.006	0.004			
Rhinolophus alcyone_(BOLD:AAD6851)	0.148	0.151	0.148	0.214	0.211	0.211		
Rhinolophus hipposideros_(BOLD:AAD0389)	0.137	0.135	0.137	0.214	0.212	0.212	0.160	
Rhinolophus pumilus_(BOLD:AAI0440)	0.172	0.169	0.172	0.242	0.239	0.236	0.173	0.200

Appendix S10. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the three Taphozous mauritianus samples from South Africa.

	UP_953	UP_712	UP_769	AAH9837
UP_953_Taphozous_mauritianus				
UP_712_Taphozous_mauritianus	0.004			
UP_769_Taphozous_mauritianus	0.008	0.004		
Taphozous longimanus_(BOLD:AAH9837)	0.146	0.146	0.146	
Taphozous melanopogon_(_ABRVN431-06_)	0.234	0.234	0.240	0.219

Appendix S11. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the four Tadarida aegyptiaca samples from South Africa.

	UP_159	UP_156	UP_157	UP_158
UP_159_Tadarida_aegyptiaca				
UP_156_Tadarida_aegyptiaca	0.002			
UP_157_Tadarida_aegyptiaca	0.002	0.000		
UP_158_Tadarida_aegyptiaca	0.004	0.002	0.002	
Tadarida teniotis_(BOLD:AAF7233)	0.227	0.227	0.227	0.229

Appendix S12. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the seven Nycticeinops schlieffenii from South Africa.

	UP_719	UP_720	UP_773	UP_775	UP_776	UP_777
UP_719_Nycticeinops_schlieffenii						
UP_720_Nycticeinops_schlieffenii	0.000					
UP_773_Nycticeinops_schlieffenii	0.017	0.017				
UP_775_Nycticeinops_schlieffenii	0.000	0.000	0.017			
UP_776_Nycticeinops_schlieffenii	0.021	0.021	0.003	0.021		
UP_777_Nycticeinops_schlieffenii	0.000	0.000	0.017	0.000	0.021	
UP_778_Nycticeinops_schlieffenii	0.000	0.000	0.017	0.000	0.021	0.000

Appendix S13. Pair wise distances of COI sequence divergences (Kimura-2 parameter) of the two Miniopterus natalensis from South Africa.

	UP_201	UP_198	JF442530.1
UP_201_Miniopterus_natalensis			
UP_198_Miniopterus_natalensis	0.007		
Miniopterus natalensis_(GenBank:_JF442530.1)	0.108	0.104	
Miniopterus schreibersii_(BOLD:AAC3658)	0.161	0.161	0.163

Appendix S14. Table demonstrating the 84 South African bat samples that produced neutralising antibodies against at least two of the challenge viruses

UP number	Species names	Dilution able to neutralise the challenge virus		
		LBV	DUVV	RABV
UP 0772	*Chaerephon ansorgei*	5	5	5
UP 0835	*Chaerephon pumilus*	5	25	5
UP 0837	*Chaerephon pumilus*	25	25	0
UP 0838	*Chaerephon pumilus*	25	25	0
UP 0839	*Chaerephon pumilus*	5	5	0
UP 1004	*Epomophorus gambianus*	5	0	5
UP 0826	*Epomophorus wahlbergi*	25	0	5
UP 1003	*Epomophorus wahlbergi*	25	0	0
UP 0827	*Glauconycteris variegatus*	5	25	0
UP 0868	*Glauconycteris variegatus*	5	125	0
UP 0912	*Hipposideros caffer*	25	25	0
UP 0948	*Hipposideros caffer*	5	5	5
UP 1013	*Insectivorous bat*	5	5	0
UP 1377	*Miniopterus natalensis*	5	0	5
UP 1380	*Miniopterus natalensis*	5	5	0
UP 1383	*Miniopterus natalensis*	5	25	5
UP 1385	*Miniopterus natalensis*	5	5	0
UP 1386	*Miniopterus natalensis*	0	625	0
UP 1389	*Miniopterus natalensis*	5	0	5
UP 1398	*Miniopterus natalensis*	5	5	0
UP 1399	*Miniopterus natalensis*	5	0	5
UP 1403	*Miniopterus natalensis*	5	5	0
UP 1406	*Miniopterus natalensis*	5	0	5
UP 1410	*Miniopterus natalensis*	5	625	0
UP 1411	*Miniopterus natalensis*	5	0	5
UP 0968	*Mops condylurus*	5	5	0
UP 0967	*Mops condylurus*	5	5	0
UP 0726	*Myotis tricolor*	5	5	0
UP 0915	*Neoromicia capensis*	5	5	0
UP 0963	*Neoromicia capensis*	5	5	0
UP 0763	*Neoromicia helios*	5	5	0
UP 0923	*Neoromicia helios*	5	5	0
UP 0931	*Neoromicia helios*	25	5	0
UP 0727	*Neoromicia nana*	5	5	0
UP 0962	*Neoromicia nana*	5	5	0
UP 0964	*Neoromicia rueppellii*	5	5	0
UP 0961	*Neoromicia zuluensis*	5	5	0

UP 0829	*Nycteris thebaica*	25	5	0
UP 0843	*Nycteris thebaica*	25	25	0
UP 0854	*Nycteris thebaica*	125	125	0
UP 0855	*Nycteris thebaica*	625	625	0
UP 0856	*Nycteris thebaica*	125	625	25
UP 0913	*Nycticeinops schlieffenii*	5	25	0
UP 0840	*Pipistrellus hesperidus*	25	5	0
UP 0842	*Pipistrellus hesperidus*	25	5	0
UP 0871	*Pipistrellus hesperidus*	625	125	0
UP 0745	*Pipistrellus rusticus*	5	25	0
UP 0927	*Rousettus aegyptiacus*	25	0	5
UP 1456	*Rousettus aegyptiacus*	125	0	5
UP 1471	*Rousettus aegyptiacus*	25	25	5
UP 1483	*Rousettus aegyptiacus*	125	25	5
UP 1487	*Rousettus aegyptiacus*	25	25	0
UP 1492	*Rousettus aegyptiacus*	25	0	5
UP 1506	*Rousettus aegyptiacus*	625	5	0
UP 1511	*Rousettus aegyptiacus*	5	5	0
UP 1513	*Rousettus aegyptiacus*	25	5	0
UP 1514	*Rousettus aegyptiacus*	125	0	5
UP 1515	*Rousettus aegyptiacus*	0	5	0
UP 1517	*Rousettus aegyptiacus*	5	5	0
UP 1518	*Rousettus aegyptiacus*	0	5	5
UP 1520	*Rousettus aegyptiacus*	25	5	0
UP 1523	*Rousettus aegyptiacus*	625	5	0
UP 1531	*Rousettus aegyptiacus*	125	5	0
UP 1532	*Rousettus aegyptiacus*	625	5	0
UP 1534	*Rousettus aegyptiacus*	5	5	0
UP 1535	*Rousettus aegyptiacus*	5	0	5
UP 1539	*Rousettus aegyptiacus*	0	5	5
UP 1008	*Scatphalous spp.*	25	25	0
UP 1010	*Scatphalous spp.*	5	25	0
UP 1017	*Scatphalous spp.*	125	5	0
UP 1019	*Scatphalous spp.*	5	25	0
UP 1021	*Scatphalous spp.*	5	5	0
UP 1022	*Scatphalous spp.*	25	5	0
UP 0832	*Scotophilus dinganii*	125	5	0
UP 0845	*Scotophilus dinganii*	25	25	0
UP 0847	*Scotophilus dinganii*	5	25	0
UP 0848	*Scotophilus dinganii*	125	25	0
UP 0849	*Scotophilus dinganii*	25	5	0
UP 0852	*Scotophilus dinganii*	625	125	0

UP 0866	*Scotophilus dinganii*	5	25	0
UP 0956	*Scotophilus dinganii*	5	5	0
UP 0958	*Scotophilus dinganii*	25	25	0
UP 0959	*Scotophilus dinganii*	5	5	0
UP 0960	*Scotophilus leucogaster*	25	25	5