Epidemiological aspects of the interface of sylvatic and dog rabies in South Africa

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I, ……Ayla Janina-Bertha Malan……. declare that the dissertation, which I hereby submit for the degree……MSc Microbiology…….. at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ..……..

DATE: ……7 September 2021……

I.1 Acknowledgements

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I.2 Summary

Rabies is a viral zoonotic disease that causes an estimated 60,000 preventable human fatalities in rabies affected countries every year. While very few countries have eliminated terrestrial rabies, the burden is the highest in developing countries in Africa and Asia, where 99% of human rabies cases are caused by domestic dogs. One of these rabies-endemic countries is South Africa, where canine-mediated rabies occurs throughout the country – causing an estimated 42 preventable human deaths every year while also impacting various livestock and sylvatic species. Although canine rabies has been described for many provinces in South Africa, the effect and possible maintenance of rabies by sylvatic species is unknown and as such, this study aimed to investigate the interface of canine and sylvatic rabies in South Africa.

By using empirical rabies surveillance data collected over a 21-year period in South Africa, it was found that rabies remains endemic to canine populations throughout the country with the most cases occurring along the eastern seaboard. In contrast, our findings suggested that sylvatic rabies cases were found throughout the country, with more cases observed in rural farming communities in the northern parts of the country – specifically in the North West and Limpopo provinces. Based on this, the two provinces were selected for molecular epidemiological analyses investigating the interface between domestic and sylvatic rabies cases. The molecular epidemiological analyses relied on two gene regions (*viz.* the partial nucleoprotein gene and G-L intergenic region) and was used to not only update our current understanding of rabies within each province, but to identify unique rabies endemic cycles in sylvatic species.

The results provided strong evidence that suggests that sylvatic species from both the North West and Limpopo provinces in South Africa were able to maintain rabies endemic cycles independently from domestic dogs. More specifically, we found evidence in support of three separate endemic cycles of sylvatic rabies throughout the North West Province and one endemic cycle of sylvatic rabies in the western parts of the Limpopo Province. In addition, we also indicated genetic homology between sequences collected from dogs and sylvatic species – suggesting that spill-over infections had occurred in both provinces. Therefore, to eliminate canine-mediated rabies from South Africa by 2030, rabies within the sylvatic populations of South Africa would need to be targeted by means of oral vaccination campaigns while canine rabies is controlled, to prevent spill-over infections from sylvatic species after canine rabies has been eliminated.

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I.6 List of Abbreviations

ABLV	*Australian bat lyssavirus*
ADB	agarose dissolving buffer
AIC	Akaike's information criterion
ARAV	*Aravan lyssavirus*
ARC-OVI	Agricultural Research Council - Onderstepoort Veterinary Institute
BBLV	*Bokeloh bat lyssavirus*
BEAST	Bayesian Evolutionary Analysis by Sampling Trees
bp	base pairs
cDNA	complementary deoxyribonucleic acid
DAFF	Department of Agriculture, Fisheries and Forestry
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DUVV	*Duvenhage lyssavirus*
EBLV-1	*European bat 1 lyssavirus*
EBLV-2	*European bat 2 lyssavirus*
EC	Eastern Cape Province
EDTA	ethylenediaminetetraacetic acid
FAT	Fluorescent antibody test
FS	Free State Province
g	g force
G	glycoprotein
GARC	Global Alliance for Rabies Control
GBLV	*Gonnoruwa bat lyssavirus*
GP	Gauteng Province
HCl	hydrochloric acid
ICTV	International Committee on the Taxonomy of Viruses
IKOV	*Ikoma lyssavirus*
IRKV	*Irkut lyssavirus*
IUPAC	International Union of Pure and Applied Chemistry
KHUV	*Khujand lyssavirus*
KZN	KwaZulu-Natal Province
L	large RNA-dependant RNA polymerase
LBV	*Lagos bat lyssavirus*
LLEBV	*Lleida bat lyssavirus*

LP	Limpopo Province
M	matrix
MCMC	Markov Chain Monte Carlo
MEGA	Molecular Evolutionary Genetics Analysis
MgCl2	magnesium chloride
µl	micro litre
ml	millilitre
mM	millimolar
MOKV	*Mokola lyssavirus*
MP	Mpumalanga Province
N	nucleoprotein
NC	Northern Cape Province
nt	nucleotide
NTD	neglected tropical disease
NW	North West Province
OIE	World Organisation for Animal Health
OR	odds ratio
ORV	oral rabies vaccine
P	phosphoprotein
PCR	polymerase chain reaction
pmol	picomole
PV	Pasteur virus
RABV	*Rabies lyssavirus*
RNA	ribonucleic acid
SA	South Africa
SHIBV	*Shimoni bat lyssavirus*
Tris	trisaminomethane
TWBLV	*Taiwan bat lyssavirus*
U	Units
UN SDGs	United Nations Sustainable Development Goals
USA	United States of America
UV	Ultraviolet
WC	Western Cape Province
WCBV	*West Caucasian bat lyssavirus*
WHO	World Health Organization

Chapter I

Literature review and study overview

1.1 Taxonomy of the *Lyssavirus* genus

Rabies is a viral zoonotic disease caused by various members of the *Lyssavirus* genus belonging to the *Rhabdoviridae* family in the order *Mononegavirales*. The *Mononegavirales* order contains negative-sense ribonucleic acid (RNA) viruses that are single stranded, non-segmented, and linear (Amarasinghe *et al.*, 2017). Based on the most recent published report, the International Committee on the Taxonomy of Viruses (ICTV, 2020) recognises 17 unique viral species in the *Lyssavirus* genus, namely: *Aravan lyssavirus* (ARAV), *Australian bat lyssavirus* (ABLV), *Bokeloh bat lyssavirus* (BBLV), *Duvenhage lyssavirus* (DUVV), *European bat 1 lyssavirus* (EBLV-1), *European bat 2 lyssavirus* (EBLV-2), *Gannoruwa bat lyssavirus* (GBLV), *Ikoma lyssavirus* (IKOV), *Irkut lyssavirus* (IRKV), *Khujand lyssavirus* (KHUV), *Lagos bat lyssavirus* (LBV), *Lleida bat lyssavirus* (LLEBV), *Mokola lyssavirus* (MOKV), *Rabies lyssavirus* (RABV), *Shimoni bat lyssavirus* (SHIBV), *Taiwan bat lyssavirus* (TWBLV), and *West Caucasian bat lyssavirus* (WCBV).

While all of the species in the *Lyssavirus* genus are causative agents for the disease rabies, the prototype member is RABV, which is not only the first lyssavirus to have been discovered (Baer, 2007) and thus the most studied species, but it also has the greatest public health impact due to its association with domestic dogs (*Canis lupus familiaris)* (WHO, 2018). While canine-mediated rabies has been eliminated from some regions and territories around the world (Lembo *et al.*, 2010; Vigilato *et al.*, 2013), the disease is still endemic to every landmass except for Antarctica and a few isolated islands (**Figure 1.1**) (WHO, 2017; WHO, 2018).

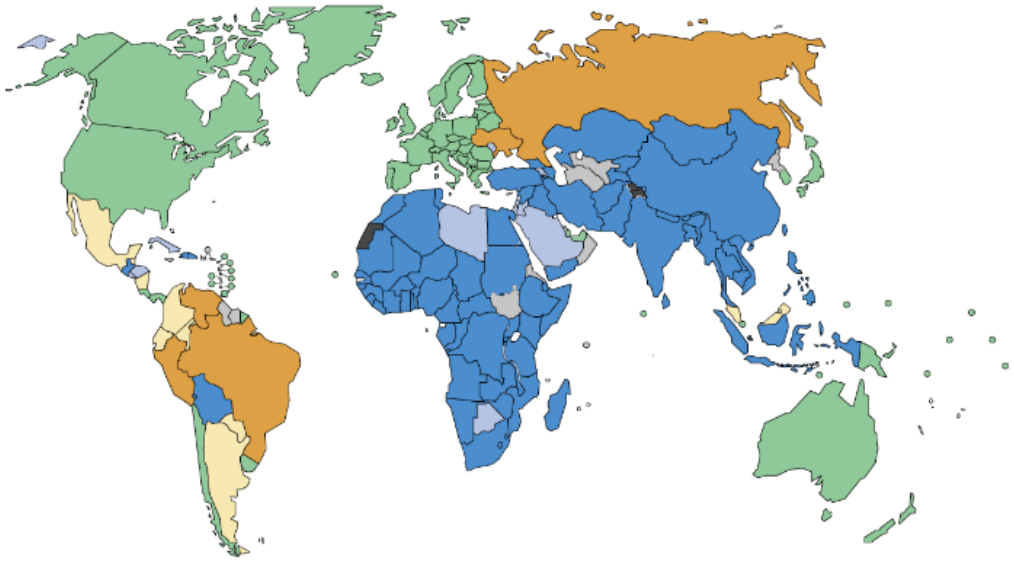

Figure 1.1: Endemicity of canine rabies and canine-mediated rabies, 2016. (Dark blue: canine rabies and canine-mediated rabies is endemic to the country; Light blue: canine rabies is endemic but there are no canine-mediated human rabies cases; Orange: a few canine rabies cases and sporadic human rabies cases; Yellow: controlled canine rabies; Green: the country is canine rabies and canine-mediated human rabies free; Grey: no information) (WHO, 2017).

1.2 Morphology and genomic structure of the *Rabies lyssavirus* genome

The RABV genome is approximately 12 kb in size and encodes five structural proteins, namely: the nucleoprotein (N) gene, the phosphoprotein (P) gene, the matrix (M) protein gene, the glycoprotein (G) gene and the large RNA-dependent RNA polymerase (L) gene (**Figure 1.2**). Situated between each of the genes is a non-coding intergenic region. These non-coding intergenic regions are usually less than five base pairs (bp) in length, except for the G-L intergenic region which is 423 bp (Tordo and Kouknetzoff, 1993) (**Figure 1.2**).

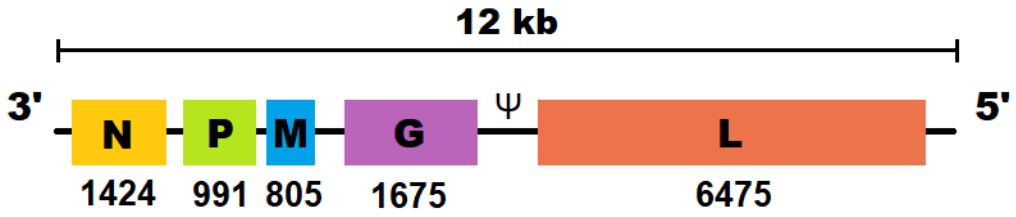

Figure 1.2: The single-stranded negative-sense RNA genome encoding five structural proteins, viz. N protein, P protein, M protein, G protein and L protein according to the Pasteur Virus (PV) strain (accession number M13215). Ψ: G-L intergenic region

At the genomic-level, the RABV particle contains a nucleocapsid core composed of gene products from the N, P and L genes that are arranged in a helical structure that is surrounded by a host-derived lipoprotein envelope. The M proteins, in turn, form a layer between the lipoprotein envelope and the nucleocapsid, while the G proteins extend from the lipoprotein envelope to form the surface projections on the virion (Wunner and Conzelmann, 2013).

1.3. The estimated burden of dog-transmitted rabies

In light of its near global distribution, rabies still causes an estimated 59,000 human deaths annually (Hampson *et al.*, 2015), with the greatest burden in Africa and Asia where 99% of human rabies cases are associated with bites from domestic dogs (Fahrion *et al.*, 2017; WHO, 2018). The estimated number of human rabies is, however, thought to still be largely underestimated across rabies-endemic countries due to under-reporting (Nel, 2013; Hampson *et al.*, 2015; Scott *et al.*, 2017). Indeed, it has been suggested that only 3% of human rabies cases are reported by health care officials, which ultimately leads to an underestimation of the true disease burden (Knobel *et al.*, 2005; Hampson *et al.*, 2015). In addition to a lack of data reporting, the number of human rabies cases in resource limited countries is often underestimated due to the use of clinical diagnoses, which relies solely on the manifestation of clinical symptoms (Mallewa *et al.*, 2007; Hampson *et al.*, 2015). The clinical diagnosis of rabies is considered highly inaccurate as the symptoms that manifest are often similar to those

observed with other encephalitic diseases such as meningitis and cerebral malaria – in-turn often leading to rabies fatalities being misdiagnosed (Cohen *et al*., 2007a; Mallewa *et al*., 2007; Hampson *et al.*, 2015; WHO, 2018).

Although the estimated number of human casualties might seem insignificant when compared to some other diseases such as malaria, rabies is the only vaccine preventable neglected tropical disease (NTD), making it the strongest and most feasible candidate for elimination under the United Nation's Sustainable Development Goals (UN SDGs) (United Nations, 2020).

1.4 The control and elimination of canine-mediated rabies

The control and elimination of canine-mediated rabies relies primarily on the vaccination of a significant proportion of the at-risk dog population to be effective (WHO, 2018). To this end, it is widely considered that dog vaccination campaigns need to reach approximately 70% of the dog population to interrupt disease transmission (WHO, 2018). This approach has been shown to effectively interrupt disease transmission and, in so doing, eliminate rabies transmission from dogs to humans (WHO, 2019). The most recent success using this strategy occurred in Latin America where the implementation of mass dog vaccination campaigns resulted in the drastic reduction of rabies cases in most countries and even elimination of canine-mediated rabies in some countries (Vigilato *et al.*, 2013).

1.5 The control and elimination of sylvatic rabies

Various sylvatic species are implicated in the transmission of rabies across the world. Rabies cases in sylvatic species are defined according to host species' ability to transmit RABV and are categorised as either sylvatic reservoir species, vector species or dead-end hosts. Sylvatic reservoir species are defined as a species that can maintain virus transmission and circulation within a population while a vector species is able to transmit RABV, but cannot maintain virus circulation within a population (Gilbert and Chipman, 2020). Furthermore, some species cannot maintain or transmit RABV and are known as dead-end hosts. Various methods to control sylvatic rabies have been used in the past (such as culling and gassing of dens), however, vaccination of susceptible populations through the use of oral rabies vaccines (ORVs) have been found to be most effective at controlling and eliminating rabies in sylvatic species (Mähl *et al.*, 2014).

1.6 Rabies lyssavirus reservoir species in countries where canine-mediated rabies had successfully been eliminated

The successful elimination of canine-mediated rabies from many rabies-endemic countries (e.g., Japan, South Korea, Australia, New Zealand) resulted in the countries self-declaring freedom from canine-mediated rabies by means of dog vaccination (WHO, 2018). In contrast, the successful control and elimination of canine-mediated rabies in other countries (e.g. the United States of America, Canada and specific European countries) enabled the countries to self-declare freedom from canine-mediated rabies by means of dog vaccination, while observing an increase in the transmission of rabies in various terrestrial sylvatic species (WHO, 2018).

1.6.1 Sylvatic rabies in Europe

While canine-mediated rabies has been eliminated from 12 European countries (Austria, Belgium, Czech Republic, Estonia, Finland, France, Germany, Italy, Luxembourg, Switzerland and the Netherlands (Cliquet, Picard-Meyer and Robardet, 2014)) to date, sylvatic rabies still poses a significant problem across western Europe where rabies transmission and persistence is primarily associated with raccoon dogs (*Nyctereutes procyonoides*), red foxes (*Vulpes vulpes*) and other sylvatic species (Holmala and Kauhala, 2006). Although various oral rabies vaccines (ORVs) had been used to successfully eliminate rabies in various wildlife species since the 1970s, the disease is still maintained within specific wildlife populations in parts of Europe (Wandeler, 1988; Mähl *et al.*, 2014). Although the history of rabies in Europe is well documented, only a few countries where canine-rabies had been eliminated, and sylvatic rabies subsequently persisted, will be discussed in detail below.

1.6.1.1 Germany

Canine rabies cases in Germany had been reported since the 1700s, with reports of rabies outbreaks in fox and wolf populations in south Germany dating back to the early 1800s (Müller *et al.*, 2004, 2012). Implementation of control measures (e.g. elimination of stray dogs, placing dogs in quarantine) to combat canine rabies in 1880 resulted in the disappearance of canine rabies cases throughout most of Germany until 1939 when the country was considered free from canine rabies (Müller *et al.*, 2004, 2012). The elimination of canine rabies did, however, not impact the persistence of sylvatic rabies, and by 1947 the fox rabies epidemic entered northern Germany from Poland, after which it spread throughout the country (Taylor, 1976; Müller *et al*., 2012). In 1977, West Germany implemented vaccination strategies towards the control of sylvatic rabies (Müller *et al.*, 2012). In contrast, East Germany implemented

measures to reduce the fox populations by means of culling of foxes and gassing of dens. However, these methods, as seen elsewhere, were found to be ineffective. Following the success of ORV campaigns targeting the sylvatic population in Switzerland; West Germany implemented the first ORV campaign in 1983. Following the success thereof, ORV campaigns were implemented throughout West Germany by 1987 (Schneider and Cox, 1983; Wachendorfer *et al.*, 1986; Müller *et al.*, 2012), while ORV campaigns in East Germany were only started in 1989. By 1991, ORVs became the preferred method for eliminating sylvatic rabies until Germany was declared free from terrestrial rabies in 2008 (Müller *et al.*, 2012).

1.6.1.2 Belgium

Between 1856 and 1860, two to six human rabies cases were reported annually even though the historic incidence of rabies within the country is not well described. In 1902 canine vaccination campaigns were implemented and no canine-mediated rabies cases were reported after 1922. The next mention of rabies within the country occurred in 1966 when fox rabies entered Belgium from Germany and subsequently spread throughout the country. After this introduction, a severe rabies outbreak occurred between 1982 and 1989 in which high numbers of sylvatic rabies cases were reported, with most of the cases occurring in the red fox populations. Rabies cases were also seen in cattle, sheep, and domestic cats during this outbreak. Methods to control fox rabies (gassing of dens and culling of foxes) were implemented in 1967. These methods proved to be ineffective at reducing the spread of rabies and ORV campaigns were implemented to curb the spread of the disease. Through rigorous ORV campaigns, wildlife rabies was eliminated in Belgium by 2000 (Aubert *et al.*, 2004; King *et al.*, 2004).

1.6.1.3 The Netherlands

No anecdotal evidence of rabies in the Netherlands exists prior to 1822, but the first canine-mediated human rabies case in the country was reported in 1843. Thereafter the disease continued to spread within the Netherlands until control measures were implemented in 1875 and rabies was thought to have been eliminated in 1923 by means of dog vaccination campaigns. However, rabies re-emerged in wildlife species in 1974 when it was introduced into the country after it had spread westward from Germany. In anticipation of the rise in sylvatic rabies cases, 30,000 dogs were vaccinated in and around South Limburg – a town situated to the south of the Netherlands. Despite these efforts, a rabid fox was discovered in the Groningen Province situated to the North of the country in 1974. After this discovery, ORV

campaigns directed at targeting sylvatic rabies were implemented until 1991 when the Netherlands was declared free from terrestrial rabies (Aubert *et al.*, 2004).

1.6.1.4 Luxembourg

Anecdotal evidence suggests the presence of canine rabies in Luxembourg since at least 1830. Canine rabies then spread throughout the country with the last cases recorded in 1912. The introduction of sylvatic rabies occurred at the same time the disease was introduced into neighbouring Belgium (1966) and control measures were rapidly implemented. After gassing and culling of the local fox populations proved ineffective, authorities in Luxembourg made dog vaccinations compulsory to prevent a spill-over of sylvatic RABV into the dog populations. The sylvatic rabies outbreak continued to spread throughout the country with 57% of all reported cases belonging to red foxes, followed by roe deer, badgers and stone martens (Wolff and Frisch, 1985; Aubert *et al.*, 2004). Joint ORV campaigns indirectly covering Luxembourg were conducted by Belgium, France and Germany in 1986 and 1987 as most of Luxembourg lies in close proximity to these countries (Brochier *et al.*, 1988). Bi-annual ORV campaigns were implemented within Luxembourg in 1988 which ultimately led to the elimination of sylvatic rabies in 1999 (Aubert *et al.*, 2004).

1.6.1.5 France

Rabies has been documented in France since the Middle Ages, with domestic dogs being responsible for the spread of most rabies cases within the country during that period. Following the death of 24 people in Paris in 1878, French authorities implemented control measures which resulted in the culling of 4,000 dogs in the city (Blancou, 2004). France eventually became rabies free in 1960 following canine vaccination and the destruction of stray dogs, however, this status was short-lives as the fox rabies epidemic had spread from Poland into France by 1968 (Sacramento *et al.*, 1992; Abellán García *et al.*, 2004). Vaccinations of domestic animals soon ensued and by 1972 the number of cattle rabies cases had decreased dramatically where they previously increased as the number of fox rabies cases increased (Aubert *et al.*, 2004). In 1986 France implemented its first ORV campaign in collaboration with Belgium, Luxembourg and Switzerland, and was declared free from terrestrial rabies in 2001 (Mähl *et al.*, 2014). France, however, temporarily lost that status after a dog with rabies was imported into the country in 2008, but regained its rabies free status in 2010 by means of vaccination campaigns (Freuling *et al.*, 2013).

1.6.1.6 Switzerland

No evidence of rabies in Switzerland exists prior to 1803. However, it is noted that fox rabies was widespread throughout eastern Switzerland by 1819 (Köchlin, 1835). Although canine rabies was already present within the country by that time, it is not known when or from where the canine variant first entered Switzerland. Improved diagnostic capabilities and surveillance systems in 1903 resulted in a decrease in the actual number of reported cases and no rabies cases were reported in Switzerland after 1928. The first mention of rabies thereafter came when the fox rabies epidemic had reached Switzerland by 1967. As observed elsewhere, disease transmission was interrupted through the use of ORV campaigns and Switzerland was declared rabies free in 1999.

1.6.1.7 Sweden

Rabies in Sweden can be traced back to the Middle Ages where the disease was prevalent in domestic dogs and wolves. In 1824, an outbreak of rabies in domestic dogs occurred in Stockholm, which subsequently spread throughout the country until the disease had become endemic to Sweden by 1857 (Westerling *et al.*, 2004). However, through the implementation of control measures and their subsequent success, Sweden has been free from rabies in domestic dogs for more than a hundred years (Berndtsson *et al.*, 2011). To date, the raccoon dog remains a major reservoir for sylvatic rabies cycles in Sweden as fox densities are thought to be too low to allow rabies endemic cycles to be sustained (Holmala and Kauhala, 2006; Mähl *et al*., 2014).

1.6.2 Sylvatic rabies in the United States of America

Anecdotal evidence suggests that canine rabies has been present in the United States of America (USA) since the early 1700s (Rupprecht *et al.*, 1995), while fox rabies was first introduced into the western regions of the USA during the 1800s through the importation of foxes from England for fox-hunting. In an effort to control rabies in the dog population, mass dog vaccination campaigns and the culling of stray dog populations were implemented. These approaches worked and canine rabies cases started to decline in 1920 and canine-mediated rabies was eliminated from the dog populations by 1970 (Rupprecht *et al.*, 1995; Velasco-Villa *et al.*, 2008). During this time, the occurrence of sylvatic rabies cases had far exceeded those seen in domestic dogs (Smith, 1996), and sylvatic rabies had spread to the eastern USA by the 1950s (Rupprecht *et al.*, 1995). Rabies had become enzootic to the coyote (*Canis latrans*) and gray fox (*Urocyon cinereoargenteus*) populations by the late 1980s, respectively. This outbreak was contained through extensive ORV campaigns, which also saw the elimination of

rabies in coyotes within these regions by 2004 (Velasco-Villa *et al.*, 2008). After canine rabies had been eliminated in the USA, cases of rabies in dogs was still reported and upon further investigation officials found the source of these to be transmission from sylvatic reservoir species (Smith, Orciari and Yager, 1995). To-date, sylvatic rabies is most prominently found in raccoons (*Procyon lotor*), skunks (*Mephitis mephitis*) and American red foxes (*Vulpes vulpes fulvus*) in the USA (Smith *et al.*, 1995; Finnegan *et al.*, 2002; WHO, 2018).

1.7 The epidemiology of rabies on the African continent

At the time of writing, no country or region in Africa was free from canine-mediated rabies with domestic dogs causing an estimated 25,000 human fatalities due to rabies every year (Hampson *et al.*, 2015). In addition to canine-mediated rabies, sylvatic species such as mongoose (yellow mongoose, *Cynictus penicillata* and slender mongoose, *Galerella sanguinea*), jackals (black-backed jackal, *Canis mesomelas* and side-striped jackal, *Canis adustus*), the Ethiopian wolf (*Canis simensis*), bat-eared foxes (*Otocyon megalotis*), and the African wild dog (*Lycaon pictus*) have also been shown to maintain rabies transmission (Bingham *et al.*, 1999; Cohen *et al*., 2007; Hughes and Macdonald, 2013; Czupryna *et al.*, 2016). These sylvatic species, however, have a seemingly negligible public health impact and are thus currently of little concern in terms of the elimination of human rabies (WHO, 2018).

On a genetic level, there are three lineages of RABV circulating on the African continent. The first lineage, referred to as the Africa 1 lineage, is the most similar to the greater "Cosmopolitan canine variant lineage" that originated from the Palearctic region which included the Middle East, North Africa, and parts of Europe (Kissi *et al.,* 1995). Despite originating in the Palearctic region, anecdotal evidence suggests that the southward dissemination of the virus through Africa can be attributed to the movement of European settlers and their companion animals throughout the continent (Smith *et al.*, 1992; Swanepoel *et al.*, 1993; Nel and Rupprecht, 2007). To date, the Africa 1 lineage primarily infects dogs, the side-striped jackal, black-backed jackal and bat-eared foxes (Davis *et al.*, 2007) throughout the African continent (**Figure 1.3**). In addition to being maintained within members of the *Canidae* family across Africa, the greater kudu (*Tragelaphus strepsiceros*) in Namibia has also been shown to maintain the Africa 1 lineage (Swanepoel *et al*., 1993; Scott *et al.*, 2013). The maintenance of RABV within a herbivorous reservoir species is unique to the Namibian kudu population as herbivorous hosts are usually considered to be dead-end hosts (Scott *et al.*, 2013). Despite the vast geographical distribution, the maintenance of the Africa 1 lineage has been shown to be largely dependent on the geographical localisation of the various respective reservoir species (Sabeta, Bingham and Nel, 2003).

Figure 1.3: Map of Africa showing the distribution of the Africa 1 lineage of RABV. The Africa 1 lineage is distributed throughout most African countries (shown in yellow) (Davis *et al.*, 2007). The Africa 1-a lineage is mainly found in northern and eastern Africa, while Africa 1-b predominates in the eastern, southern and central regions of Africa (Hayman *et al.*, 2011). Recent data for Western Sahara could not be found.

The two remaining lineages that are unique to Africa (Africa 2 and Africa 3 lineages) evolved separately and most likely have different progenitor viruses (Kissi *et al*., 1995). The Africa 2 lineage is maintained in domestic dog populations in multiple countries throughout West and Central Africa (Kissi *et al*., 1995; Hayman *et al*., 2011; Talbi *et al.*, 2009) (**Figure 1.4**).

Figure 1.4: Map showing the confirmed distribution of the Africa 2 lineage of RABV (shown in red).

The Africa 3 lineage, commonly referred to as the mongoose variant of RABV, is found only in the *Herpestidae* family in southern Africa (Kissi *et al.*, 1995; Nel *et al.*, 2005). To date, this lineage has been found to persist in the yellow and slender mongoose populations within southern African countries, such as South Africa, Namibia, Botswana and Zimbabwe (**Figure 1.5**).

Figure 1.5: Map showing the confirmed distribution of the Africa 3 lineage of RABV (shown in green) (Kissi *et al*., 1995; Nel *et al.*, 2005). Although rabies is endemic to Lesotho and Eswatini in southern Africa, the presence of the Africa 3 in these two countries is not known.

1.8 Reservoir species in southern Africa

1.8.1 Angola

The first rabies case in Angola was confirmed in 1928, after the disease had spread into the country from Zambia. The disease was mostly encountered in dogs, and very few cases were noted in wildlife and domestic animal species (Swanepoel *et al.*, 1993). The start of the Angolan civil war (1975 – 2002) has hampered the monitoring, control and elimination of the disease in the country and as a result very little information about the current epidemiology of rabies is known (Nel and Rupprecht, 2007).

1.8.2 Zambia

Anecdotal evidence suggests the presence of RABV in Zambia since 1901, but the first case in the country was only confirmed in 1913 (Swanepoel *et al*., 1993; Nel and Rupprecht, 2007). Apart from the cases of rabies in dogs, rabies cases in cattle were also reported – especially in areas bordering nature reserves where higher densities of jackal populations could be found (Swanepoel *et al.*, 1993). Between 1985 and 2004, 69.7% of all rabies samples tested within the country came from domestic dogs while only 4.5% were attributed to wildlife species (Munang'andu *et al.*, 2011). Outbreaks of rabies in cattle near the Kafue flats coincided both seasonally and geographically with outbreaks observed in the jackal populations. Previous studies have, however, suggested that domestic dogs were most likely responsible for the introduction of rabies into the cattle populations because of the low jackal population densities in the affected areas (Munang'andu *et al.*, 2011).

1.8.3 Malawi

Rabies has been endemic in Malawi since the 1920s and still remains a problem in dogs, jackals, and various other wildlife species in the country to date (Nel and Rupprecht, 2007). Between 1986 and 1992, domestic dogs accounted for 83% of all reported cases while rabies cases in jackals accounted for only 2.2% of the cases. The remainder of the cases were detected in cattle, hyenas, and other domestic and sylvatic species. Between 2015 and 2018 a total of 47 dog rabies cases were recorded, but only one wildlife case was recorded during the same period (Global Alliance for Rabies Control, 2020).

1.8.4 Mozambique

Rabies was first detected in Mozambique in 1952 after the disease spread from the Limpopo Province (LP) in South Africa (previously known as northern Transvaal). Thereafter, the disease spread rapidly within dog populations in the eastern and southern parts of the country. During an investigation of the molecular epidemiology of rabies in Mozambique, it was found that rabies cases detected in Zimbabwe and South Africa were genetically homologous to those detected in Mozambique (Coetzer *et al*., 2017a, 2019), highlighting the porous nature of the African borders and the transboundary movement of infected individuals (Zulu *et al*., 2008). Between the years 2015 and 2020, a total of 54 dog rabies cases were reported, while only 5 cases in wildlife was reported during the same time period (Global Alliance for Rabies Control, 2020).

1.8.5 Zimbabwe

The first anecdotal reference to rabies in Zimbabwe – a country that shares political borders with South Africa, Botswana, Zambia, and Mozambique – dates back to 1902 following an outbreak of canine-mediated rabies in the neighbouring country of Zambia (Swanepoel *et al*., 1993; Bingham *et al*., 1999a). Recognising this as a major health issue, the outbreak was contained in 1907 by placing taxes on dog owners after muzzling was found to be an ineffective approach (Shone, 1962). These measures proved to be effective and rabies appeared to be eliminated from the dog population in Zimbabwe by 1913. Subsequently, rabies was not detected in domestic dogs in Zimbabwe until the 1950s after the virus was re-introduced to the country (Bingham *et al*., 1999a). It is believed that the second introduction of rabies into Zimbabwe occurred as a result of the transboundary movement of infected animals between Zimbabwe and either South Africa or Botswana in the 1950s (Bingham *et al*., 1999). Despite efforts to control and eliminate the disease from the country after the second introduction, rabies spread throughout Zimbabwe and has been endemic ever since (Bingham *et al*., 1999; Sabeta *et al*., 2003; Zulu *et al*., 2008; Coetzer *et al*., 2019).

Land distribution in Zimbabwe is divided into four categories: protected areas (national parks and wildlife areas), communal farming settlements, commercial farming areas and urban settlements (Bingham *et al*., 1999a). Each of the sectors, in turn, govern which types of reservoir species are primarily found within them.

The rural communal farming areas house 51.4% of Zimbabwe's human population and 71.3% of the domestic dog population (Sabeta *et al*., 2003). As a result of the high dog population densities, previous investigations found that 56% of Zimbabwe's rabies-cases detected between 1985 and 1996 occurred within these rural areas (Bingham *et al*., 1999). Even though the dogs in the subsistence farming sector had owners, most were free-roaming dogs and the high density of the domestic dog populations in the communal areas lead to the interaction of dogs with wild carnivores in areas that shared boundaries with wildlife reserves and parks (Butler *et al.*, 2004). Previous investigations elsewhere in Africa found that the movement of dogs into wildlife areas were most likely as a result of increasing human populations that were expanding into these protected areas, such as nature reserves and game farms (Cleaveland, 1998). In these instances, it is believed that the predation of infected dogs by wild carnivores leads to disease transmission and can result in epidemics within the sylvatic species (Hughes and Macdonald, 2013).

In contrast, urban settlements were historically not associated with significant rabies outbreaks with only 13% of Zimbabwe's rabies-cases detected between 1985 and 1996 occurring within the urban areas (Bingham *et al*., 1999). In further support of the fact, only two rabies outbreaks

have been recorded in Zimbabwe's urban areas: the first (a self-limiting outbreak) in the Mutare region (Bingham *et al*., 1999a) and the second an outbreak in and around Harare Metropolitan Province which was ongoing at the time of writing (Coetzer *et al*., 2019).

Lastly, the side-striped jackal and black-backed jackal species are the main reservoir species that maintain sylvatic rabies cycles in commercial farming areas. The geographical distribution of the two jackal species further defines the occurrence of sylvatic cycles in commercial farming areas with the side-striped jackal occurring predominantly in the northern regions, and the black-backed jackal occurring in the southern, central, and western regions of the country (Bingham and Foggin, 1993). In regions with low species densities (such as commercial farming areas), jackals thrive as opportunistic predators, leading to high jackal populations in these regions (Cleaveland, 1998). Anecdotal evidence suggests that dog populations from the communal areas that surrounded the commercial farming areas initially lead to rabies being introduced to the jackal populations, with the jackal populations subsequently maintaining transmission and establishing unique endemic cycles of sylvatic rabies (Cleaveland, 1998; Bingham *et al*., 1999b). In Zimbabwe specifically, the public health impact arising from sylvatic rabies cases is very low, with previous investigations showing that only 31% of Zimbabwe's rabies-cases detected between 1985 and 1996 occurred within these commercial farming areas (Bingham *et al.,* 1999; Cohen *et al*., 2007). A total of 721 positive dog rabies cases were reported between 2015 and 2020 in Zimbabwe, while there were 36 positive cases in wildlife and 334 positive cases in livestock (Global Alliance for Rabies Control, 2020).

1.8.6 Botswana

The first confirmed case of dog rabies in Botswana dates back to 1919 (Johnson *et al.*, 2004). An outbreak of rabies in the north-western part of the country in 1950 led to the rapid spread of the disease to the East and South of the country (Swanepoel *et al*., 1993; Nel and Rupprecht, 2007). From there the disease spread into Zimbabwe and northern South Africa with dogs, cattle, jackals, and other livestock affected in the outbreak. Another outbreak occurred in western Botswana in 1980 which spread east and southwards into the Northern Cape Province (NCP) of South Africa where rabies was confirmed in the spotted hyena (Swanepoel *et al.*, 1993). Frequent mass canine vaccinations within the country had resulted in very few human rabies cases being reported. To date, the main species implicated with rabies transmission within Botswana are the domestic dog, the black-backed jackal, yellow mongoose and small-spotted genet (*Genetta genetta*) (Johnson *et al.*, 2004), while the highest number of recorded rabies cases can be found in livestock, which make up about 70% of the total rabies cases within the country (Johnson *et al.*, 2004).

1.8.7 Namibia

The first rabies case in Namibia was reported during 1906, even though its presence was already detected in 1887. It is believed that the disease was introduced into Namibia from south Angola during the 1920s, however the cross-border transmission could only be confirmed in 1938 from a dog suspected of having rabies (Hübschle, 1988; Swanepoel *et al.,* 1993; Scott *et al.*, 2016). Thereafter the disease spread southwards from the northern communal areas resulting in the spill-over of the disease into many wildlife and domestic species, with the species subsequently maintaining independent rabies cycles until rabies had become endemic to Namibia by the 1970s (Nel and Rupprecht, 2007; Scott *et al*., 2016; Athingo *et al*., 2020). At the time of writing, dog rabies cases were mostly seen in the northern communal areas of the country where the highest human population density was found. However, based on laboratory confirmed rabies cases, black-backed jackals are believed to maintain RABV cycles in the Etosha National Park (Bellan *et al.*, 2012). In contrast, kudu and jackal populations are believed to maintain rabies transmission in the central regions of the country as cattle fences do not prevent the movement of these reservoir species between regions, while other wildlife rabies cases (such as the African wild cat, caracal, genet and mongoose) are routinely detected in the southern arid regions of the country (Swanepoel *et al.*, 1993; Courtin *et al.*, 2000; Scott *et al.*, 2016). From there, the disease spread into the NC Province of South Africa after the disease had been seen in bat-eared foxes in the southern regions of Namibia (Swanepoel *et al*., 1993; Scott *et al.*, 2016).

Interestingly, the greater kudu populations of Namibia are the only herbivorous species on the African continent to maintain RABV cycles. The first rabies case in kudu dated back to 1975 but it was not until 1977 that a sharp increase in the number of rabies cases within kudu could be seen in the western regions of central Namibia. The kudu rabies epizootic spread eastward by 1979 until much of the central region of Namibia had succumbed to kudu rabies by 1982 (Hassel, 1982; Scott, Coetzer and Nel, 2016) and persisted in the kudu populations of Namibia until 1985 (Barnard and Hassel, 1981; Swanepoel *et al.*, 1993). Thereafter, only sporadic cases in the kudu populations were seen until another outbreak within kudu occurred in 2002 and subsequently became endemic to the country (Scott *et al.*, 2012, 2016).

Rabies cases in cattle in the central regions of Namibia generally saw a rise in the number of cases after an increase in rabies cases in kudu had been observed (Schneider, 1985; Hübschle, 1988; Scott *et al.*, 2016). Spill-over infections from kudu to cattle are feasible as both these species groups are predominantly found in central Namibia where game farms and commercial farms are found (Scott, Coetzer and Nel, 2016).

1.8.8 Kingdom of Lesotho

Rabies spread into Lesotho from the KwaZulu-Natal (KZN) Province in South Africa in 1982 and the disease has remained endemic since (Swanepoel *et al*., 1993; Coetzer *et al.*, 2017b). Routine rabies surveillance has proven to be difficult because of Lesotho's mountainous terrain (Swanepoel *et al.*, 1993) and the occurrence of cross-border transmission. Indeed, molecular epidemiological analyses suggested that cross-border transmission occurred between Lesotho and three of South-Africa's provinces, *viz*. Free State (FS), Eastern Cape (EC), and KZN (Ngoepe *et al*., 2009; Coetzer *et al.*, 2017b). Between 2016 and 2020, a total of 41 positive dog rabies cases were reported, while 43 cases in livestock (and no wildlife case) were reported for the same time period (Global Alliance for Rabies Control, 2020). Previous molecular epidemiological analyses had, however, found evidence that eluded to the fact that a potential sylvatic rabies cycle had become established in the country but this observation could not be proved or substantiated due to a lack of supporting data (Coetzer, Coertse, *et al.*, 2017).

1.8.9 Eswatini

Rabies has been endemic to Eswatini (formerly known as the Kingdom of Swaziland) since 1954 when the disease spread from the Maputo district of Mozambique into the land-locked country. Between 2015 and 2020, 33 positive dog rabies cases were reported, while only three positive cases were reported in livestock and two positive wildlife case were reported for the same time period (Global Alliance for Rabies Control, 2020).

1.8.10. Rabies in South Africa

The first case of canine rabies in South Africa was reported in Port Elizabeth in 1893 when an infected dog was imported into the country from England (Swanepoel *et al.*, 1993). The subsequent outbreak of rabies in this region spread further inland and was brought under control in 1894 through the muzzling of dogs, restricting the movement of dogs and destruction of free-roaming dogs (Hutcheon, 1894; Snyman, 1940; Swanepoel *et al.*, 1993). Between 1913 and 1950, South Africa was thought to be free of canine mediated rabies, while the disease was detected in other southern African countries such as Namibia, Angola, Zambia, Botswana and Zimbabwe (Swanepoel *et al.*, 1993). The second introduction of rabies into South Africa occurred when rabies spread from either Botswana or Zimbabwe into the northern regions of South Africa in the 1950s – resulting in the establishment and maintenance of RABV in various reservoir species in the northern parts of the country. In addition to the

introduction into South Africa in the 1950s, rabies was also introduced from the neighbouring country of Mozambique on two known instances. The first introduction from southern Mozambique occurred in 1961 when the disease was introduced into the northern regions of the KZN Province (Mansvelt, 1962; Swanepoel *et al.*, 1993). This introduction lead to the subsequent outbreak of canine rabies within the province which spread along the coastal and midland regions before being brought under control in 1968 (Swanepoel *et al.*, 1993). Six years later, the disease was once again introduced into the KZN Province from Mozambique and spread within the dog populations throughout the province and into the EC Province. By 1990, the disease had become endemic to KZN, the EC and the Kingdom of Lesotho (Swanepoel *et al*., 1993; Coetzee and Nel, 2007).

Various reservoir species (domestic and sylvatic in nature) have been shown to maintain rabies across large geographical regions of South Africa. The species involved and their distribution will be discussed in detail in Chapter II.

1.9 Molecular epidemiology and the role that it plays in identifying endemic cycles of rabies

Conventional epidemiology relies on empirical burden data – generated from passive or active surveillance programmes – to predict the distribution of disease and their spread, allowing for disease control measurements to be put into place. However, if the burden data is limited, as is often the case in African countries (Hampson *et al.*, 2015), the resolution of the surveillance network is narrowed and targeted disease intervention campaigns are not feasible. While increasing the frequency and intensity of surveillance programmes would remedy this, they are often resource intensive and thus not prioritised by governmental stakeholders (Lembo *et al.*, 2010; Nel, 2013). In such instances, molecular epidemiological analyses have been shown to be a supplementary method whereby the resolution of the surveillance network can be improved (Coetzer *et al.*, 2017a, 2017b). Indeed, molecular epidemiological studies allows researchers to identify endemic cycles of a disease – in so doing gaining an improved understanding of the epidemiology of the disease whilst relying on limited surveillance data (Eybpoosh *et al.*, 2017).

To date, three gene regions are mostly used for molecular epidemiological investigations into RABV endemic cycles, namely the N gene, the P gene and the G gene (and it's adjacent G-L intergenic region) (Sacramento *et al.*, 1992; Tordo and Kouknetzoff, 1993; Nadin-Davis *et al*., 2000). However, the M and L genes are used to a lesser extent as discussed below. Using the N gene and G-L intergenic region in particular enables disease spread to be inferred, the emergence of new endemic cycles to be identified and the geographical range of existing

rabies endemic cycles to be updated. The use of molecular epidemiological investigations for RABV in Africa has been undertaken extensively with most of the studies relying on either the N gene or the G-L intergenic region to infer genetic relatedness (**Figure 1.2**) (Sacramento, Bourhy and Tordo, 1991).

1.9.1 N gene analyses

Analysis of the N gene allows for characterisation of distantly related *Lyssavirus* strains while also allowing for precise epidemiological studies to be undertaken (Bourhy *et al.,* 1992; Tordo and Kouknetzoff, 1993). Furthermore, the N gene shows more sequence homology compared to that of the G-L intergenic region and can thus be used for comparison over long evolutionary periods – making the N gene ideal for molecular clocking and evolutionary inferences (Bourhy *et al*., 1992, 1993).

1.9.2 G gene and G-L intergenic region analyses

In contrast to the N gene, the G-L intergenic region is highly variable as there is limited selective pressure upon this region of the viral genome, enabling it to act as a neutral indicator of viral evolution as genetic changes observed in this gene region most likely results from random genetic drift events as opposed to natural selection acting upon the gene region (Sacramento, Bourhy and Tordo, 1991). To date, this region has been used most extensively for molecular epidemiological analyses in southern Africa (Sabeta *et al.*, 2003; Zulu *et al*., 2009; Coetzer *et al.,* 2017a; Coetzer *et al.*, 2017b; Coetzer *et al.,* 2019).

1.9.3 P gene analyses

The P gene is the least conserved gene region of the RABV genome and is therefore highly variable among *Lyssavirus* strains (Tordo and Kouknetzoff, 1993). Previous findings have suggested that phylogenetic analyses using this gene region provided similar results to that seen using the N gene and the G-L intergenic region (Kobayashi *et al.*, 2007).

1.9.4 M gene analyses

The M gene conservation depends on how closely related the lyssaviruses under investigation are. Between closely related lyssaviruses this gene region is the least conserved of all gene regions, while it is highly conserved in distantly related lyssaviruses (Tordo and Kouknetzoff, 1993).

1.9.5 L gene

The L gene is the most conserved gene region among the lyssaviruses and is therefore not routinely used for genetic characterisation or molecular epidemiological analyses as variations between viruses will as a rule be negligible with respect to this gene (Tordo and Kouknetzoff, 1993).

1.10 Hypothesis

Based on evidence seen in the USA and selected western European countries where the persistent transmission of sylvatic rabies had been observed in the absence of canine-mediated rabies, we hypothesise that sylvatic species within South Africa are not only capable of maintaining rabies endemic cycles independently from rabies cycles in dogs but are also, in theory, capable of re-introducing the disease into immunologically naïve dog populations. This would suggest that the persistence of rabies in sylvatic species could, in theory, hinder the country's self-declaration of freedom from dog-mediated rabies by means of vaccination (OIE, 2020), while also suggesting that rabies in sylvatic populations would have to be specifically targeted for rabies elimination if dog-mediated rabies is to be completely eliminated from the country.

1.11 Aim

The aim of this study was to gain an improved understanding of the genetic relationship between RABV sequences obtained from sylvatic and canine species within the Limpopo (LP) and North West (NW) provinces of South Africa. To this end, we undertook a molecular epidemiological analysis of both the G-L intergenic region and the N gene for rabies-positive samples sourced from within South Africa and its neighbouring countries.

1.12 Objectives

- Undertake a molecular epidemiological analysis of the RABV G-L intergenic region from rabies-positive animals originating from the LP and NW provinces within South Africa in context to sequences sourced from neighbouring provinces.
- Undertake a molecular epidemiological analysis of the partial N gene region of samples collected from rabies-positive animals originating from the LP and NW provinces within South Africa in context to sequences sourced from neighbouring provinces

Chapter II

Epidemiology of rabies in South Africa,

1998 - 2019

2.1 Introduction

South Africa is a country situated at the southern-most tip of the African continent and is further divided into nine administrative provinces, *viz*. the LP, NW, NC, EC, KZN, FS, Western Cape (WC), Mpumalanga (MP), and Gauteng (GP) provinces (**Figure 2.1**). In addition, South Africa shares political borders with Namibia, Botswana, Zimbabwe, Mozambique, Lesotho and Eswatini (previously known as the Kingdom of Swaziland).

Figure 2.1: Map of South Africa and its nine provinces (Fairbanks *et al.*, 2000)

While records suggests that rabies may have been present in South Africa for more than a century (Swanepoel *et al.*, 1993), six of the nine South African provinces (*viz*. LP, NW, MP, KZN, EC, and FS) are currently considered endemic for canine-mediated rabies. In contrast, the three remaining provinces (NC, WC, and GP) have been shown to only experience sporadic cases and outbreaks and are thus considered vulnerable to outbreaks but not endemic to canine-mediated rabies (Cohen *et al*., 2007b; Sabeta *et al.*, 2007; Ngoepe *et al.*, 2009; Mkhize *et al*., 2010; Sabeta *et al*., 2013; Hergert *et al.*, 2018) (**Figure 2.2A**).

In addition to canine-mediated rabies, the epidemiology of rabies in South Africa is further complicated by the occurrence and geographical distribution of various sylvatic reservoir species that are capable of maintaining and transmitting rabies. While any sylvatic mammal could, in theory, be infected with rabies, two sylvatic reservoir species (jackals and bat-eared foxes) have been shown to be capable of maintaining the canine variant of the RABV in South Africa. While jackal populations are most prominently found in subsistence and commercial farming areas, as well as bushveld areas, they appear to be the dominant maintenance host

in the northern areas of South Africa (**Figure 2.2B**) (Zulu *et al.*, 2009; Bishop *et al.*, 2010). Despite having a relatively large geographical distribution throughout South Africa, bat-eared fox populations appear to be the dominant maintenance host in the western areas of the country (Bishop *et al.*, 2010) (**Figure 2.2C**). In contrast, rabies cases in mongoose populations (capable of transmitting the mongoose variant of the RABV) are endemic to the central plateau of South Africa (**Figure 2.2D**).

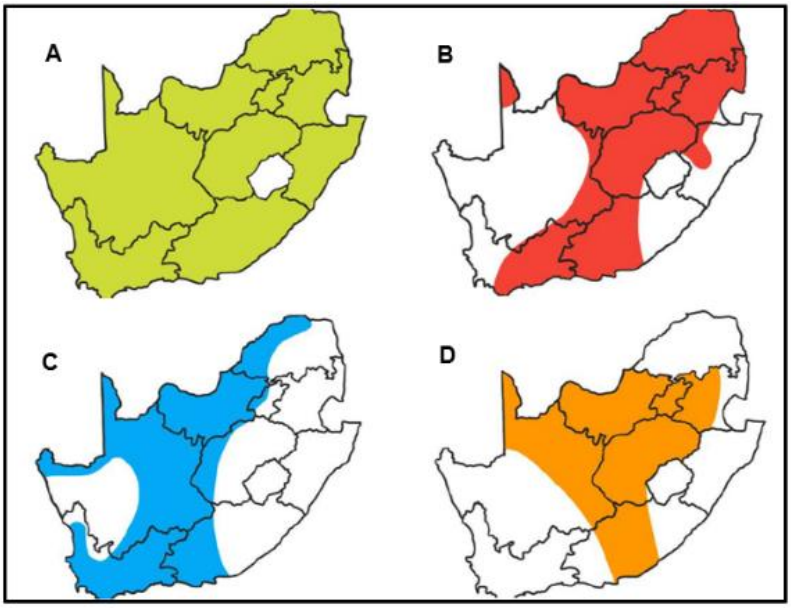

Figure 2.2: Distribution of major rabies reservoir species in South Africa. (A = domestic dogs; B = black-backed jackals; C = bat-eared foxes; D = yellow mongoose). Maps adapted using data generated by The Endangered Wildlife Trust (EWT, 2020).

Anecdotal evidence suggests that rabies-positive sylvatic species pose a negligible public health impact due to the limited interaction between humans and wildlife in general, including in South Africa (Cleaveland, 1998; Cohen *et al.*, 2007). However, sylvatic reservoir species still pose a risk of reintroducing the disease into immunologically naïve domestic dog populations where ecological niches overlap – highlighting the importance of understanding the epidemiology and transmission dynamics of endemic cycles of sylvatic rabies (King *et al.*, 2004).

Molecular epidemiological investigations can not only be used to gain an improved understanding of rabies within a particular area but can also be used to investigate the interface between canine and sylvatic rabies. In evidence of this, the epidemiology of the RABV in the LP (Cohen *et al*., 2007b; Zulu *et al.*, 2009; Sabeta *et al.*, 2011a), MP (Zulu *et al*., 2009), GP (Sabeta *et al.*, 2013), KZN (Coetzee and Nel, 2007; Shwiff *et al.*, 2016; Hergert *et al*., 2018; LeRoux *et al.*, 2018), WC (Sabeta *et al.*, 2007; Grewar, 2010), FS (Ngoepe *et al*., 2009), and the NC (Swanepoel *et al.*, 1993; Sabeta *et al.*, 2007; Weyer *et al*., 2011) provinces had been investigated to gain insights into rabies and its transmission dynamics in the country.

Despite having a large cohort of in-depth studies investigating the epidemiology of rabies at the sub-national level, a comprehensive longitudinal epidemiological investigation of rabies at the national level had, to the best of our knowledge, not been undertaken at the time of writing. As such, the aim of this chapter was to summarise the prevalence of rabies in South Africa – using empirical surveillance data collected between 1998 and 2019 – to gain an improved understanding of the epidemiological patterns of rabies within the country. In addition, we also endeavoured to identify specific provinces where the occurrence and persistence of sylvatic rabies warranted further investigation and scrutiny by means of molecular epidemiological analyses.

2.2 Materials and Methods

2.2.1. Data collection

For the purpose of this investigation, rabies surveillance data collected between 1998 and 2019 were obtained from the two laboratories in South Africa accredited in undertaking rabies diagnosis. These laboratories were the Central Veterinary Laboratory (CVL) situated in the GP province (Agricultural Research Council – Onderstepoort Veterinary Research (ARC-OVR), Rabies Unit) and the Provincial Veterinary Laboratory (PVL) in the KZN province (Allerton Provincial Veterinary Laboratory). The surveillance data, consisting of the i) year of diagnosis, ii) species subjected to diagnosis, iii) location of sampling, and iv) diagnostic outcome was used for subsequent analyses.

The geographic distribution for all positive and negative RABV cases throughout South Africa was visualised using the Tableau software package (version 2020.4.1, Seattle, USA) and relied on the use of geographic coordinates associated with each sample.

2.2.2. Statistical analyses

All the data were combined and consolidated into a single Excel spreadsheet (Microsoft Office 2016), after which a descriptive analysis was conducted for the entire dataset.

Furthermore, the species in the dataset were categorised as follows: bat (all bat species), dog (all domestic dog species), cat (all domestic cat species), livestock (bovine, caprine, equine, porcine and ovine species), mongoose (all mongoose species), and sylvatic species (all wildlife species excluding mongoose and bat species). In addition, the sylvatic species were further classified as either belonging to jackal and bat-eared fox species, or all other wildlife species (excluding mongoose and bat species).

2.3 Results

2.3.1. Overview of samples tested for rabies in South Africa, 1998 – 2019

Between 1998 and 2019, a total of 37,876 samples were subjected to rabies diagnosis, of which 31.5% (11,920) were rabies-positive (**Table 2.1**; **Table A1, Appendix materials**). Considering the rabies-positive cases specifically, most originated from dogs (56.1% of the positive cases), followed by livestock (22.2% of the positive cases), mongoose (9.29% of the positive cases), sylvatic (9.57% of the positive cases), feline (2.79% of the positive cases), and bats (0.11% of the positive cases) (**Table 2.1**, **Figure 2.3).**

Table 2.1: Rabies-positive and -negative cases in South Africa per species group, 1998 – 2019.

Species group	Positive cases	Negative cases	% Positive
Dog	6,682	12,254	35.3
Livestock	2,645	3,696	41.7
Mongoose	1,107	2,683	29.2
Sylvatic	1,141	3,436	24.9
Cat	332	3,063	9.78
Bat	13	825	1.55
Total	**11,920**	**25,956**	**31.5**

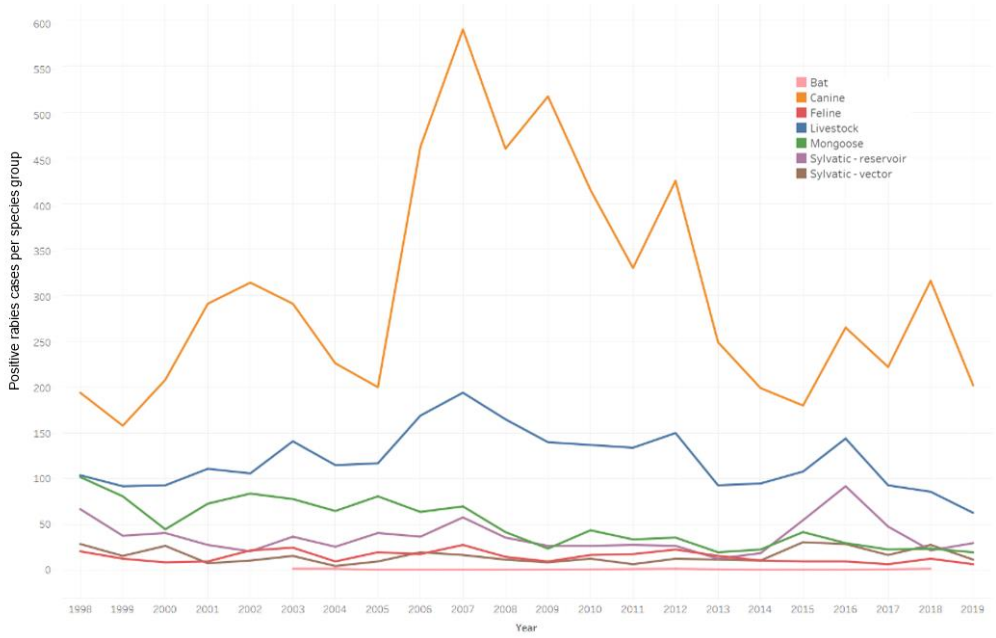

Figure 2.3: Positive rabies cases per species group per year, 1998 - 2019.

While the average number of samples subjected for diagnostic confirmation remained fairly standard across the years (Min: 1,418, Max: 2,279, Mean: 1,738) not all of the provinces contributed equally in terms of submitting samples for diagnosis. For example, between 1998 and 2019, most of the samples sent for diagnostic confirmation originated from KZN (average of 621 suspect cases per year) with the exception of one year (2009) when the most samples originated from MP (**Figure 2.4**). During the same time period, the NC contributed the least number of suspect rabies cases for diagnosis (average of 60 suspect cases per year) (**Figure 2.4**).

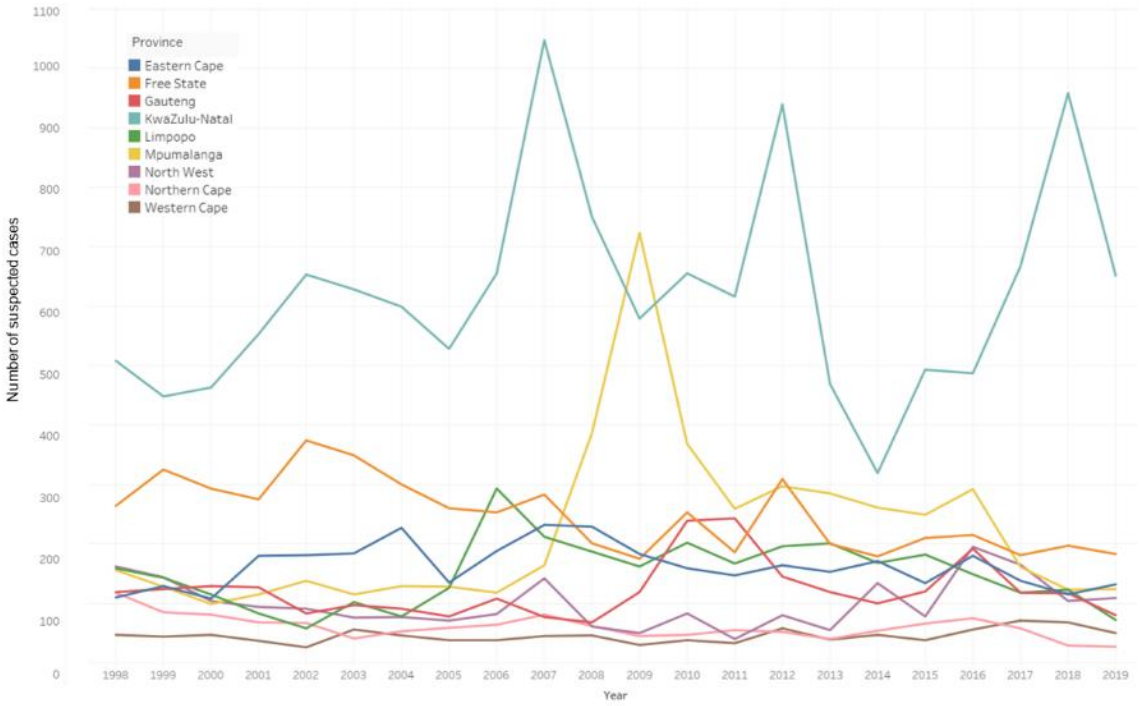

Figure 2.4: Number of samples sent for diagnostic testing, per province per year, between 1998 and 2019

2.3.2 Rabies in dogs

Between 1998 and 2019, samples originating from domestic dogs accounted for 50.0% (18,936/37,876) of all the samples subjected to rabies diagnosis in South Africa. Of those, 6,682 (35.3%) were found to be rabies positive (**Table 2.1**, **Table 2.2**). During the 21-year period, the LP and the EC provinces had the highest percentage of positive rabies cases in dogs (52.8% and 50.5% respectively), while the WC recorded the lowest percentage of rabies positive cases in dogs (2.00%) (**Table 2.2**).

Table 2.2: Suspect rabies samples collected from dogs across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	883	866	50.5
Free State	408	948	30.1
Gauteng	75	1,061	6.60
KwaZulu-Natal	3,565	6,224	36.4
Limpopo	700	627	52.8
Mpumalanga	910	1,711	34.7
North West	107	360	22.9
Northern Cape	29	210	12.1
Western Cape	5	245	2.00
Total	6,682	12,252	35.3

2.3.3 Rabies in cats

Between 1998 and 2019, 3,394 samples originating from domestic cats were sent for diagnostic testing in South Africa and 9.78% (*n = 332*) of those tested positive for rabies (**Table 2.1**, **Table 2.3**). During the 21-year period, the NC and the FS provinces had the highest percentage of positive rabies cases in cats (26.2% and 24.9% respectively), while GP recorded the lowest percentage of rabies positive cases in cats (1.57%) (**Table 2.3**).

Table 2.3: Suspect rabies samples collected from cats across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	32	162	16.5
Free State	137	427	24.9
Gauteng	6	376	1.57
KwaZulu-Natal	58	1,326	4.16
Limpopo	10	112	8.20
Mpumalanga	16	262	5.76
North West	25	173	12.6
Northern Cape	37	104	26.2
Western Cape	11	120	8.40
Total	332	3,062	9.78

2.3.4 Rabies in livestock

Between 1998 and 2019, a total of 6,341 suspect rabies samples collected from livestock were submitted for diagnostic testing, of which 41.7% (*n = 2,645*) tested positive (Table 2.1, **Table 2.4**). The EC and LP had the highest percentage of rabies positive cases in livestock (65.8% and 44.2% respectively) while the WC had the lowest percentage of rabies positive cases in livestock (5.41%) (**Table 2.4**).

Table 2.4: Suspect rabies samples collected from livestock across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	866	451	65.8
Free State	407	859	32.2
Gauteng	28	240	10.5
KwaZulu-Natal	521	728	41.7
Limpopo	331	418	44.2
Mpumalanga	225	389	36.6
North West	236	430	35.4
Northern Cape	27	111	19.6
Western Cape	4	70	5.41
Total	2,645	3,696	41.7

2.3.5 Rabies in sylvatic species other than mongooses and bats

For the 21-year period, a total of 4,577 samples collected from sylvatic species suspected of having rabies were submitted for diagnostic testing. Of those, 31.6% (*n = 1,446*) originated from jackal species and bat-eared foxes, while the remainder (*n = 3,131*; 68.4%) had been collected from other wildlife species (**Table 2.1**).

Of the 1,446 samples collected from jackals and bat eared foxes, 55.7% (*n = 806*) were confirmed as rabies-positive (**Table 2.5**), with the LP and NC provinces recording the highest percentage of positive rabies cases (74.2% and 70.8% respectively) and MP with the lowest at 12.5% (**Table 2.5**).

Table 2.5: Suspect rabies samples collected from jackal species and bat-eared foxes across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	42	31	57.5
Free State	42	59	41.6
Gauteng	32	57	36.0
KwaZulu-Natal	52	131	28.4
Limpopo	242	84	74.2
Mpumalanga	8	56	12.5
North West	87	95	47.8
Northern Cape	165	68	70.8
Western Cape	136	59	69.7
Total	806	640	55.7

For wildlife other than jackals, bat eared foxes, mongooses and bats, a total of 3,131 suspect rabies samples were submitted for diagnostic testing. Of these, only 10.7% (*n = 334*) tested

positive (**Table 2.6**). The NC had the highest percentage of rabies cases (34.8%) while only 2.77% of suspect rabies cases that originated from KZN tested positive (**Table 2.6**).

Table 2.6: Suspect rabies samples collected from wildlife other than jackals, bat-eared foxes, mongooses and bats across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	22	221	9.05
Free State	82	481	14.6
Gauteng	9	263	3.31
KwaZulu-Natal	15	526	2.77
Limpopo	41	255	13.9
Mpumalanga	16	509	3.05
North West	22	174	11.2
Northern Cape	109	204	34.8
Western Cape	18	164	9.89
Total	334	2,797	10.7

2.3.6 Rabies in members of the Herpestidae family

Between 1998 and 2019, a total of 3,790 samples originating from various mongoose species were submitted for diagnostic testing. Of these, 29.2% (*n = 1,107*) tested positive (**Table 2.1**, **Table 2.7**). The FS and NW provinces accounted for the highest percentage of rabies cases originating from mongoose samples (41.4% and 27.9 % respectively) while only 7.27% of cases originating from mongoose in LP tested positive (**Table 2.7**).

Table 2.7: Suspect rabies samples collected from mongoose species across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	68	179	27.5
Free State	623	882	41.4
Gauteng	25	293	7.86
KwaZulu-Natal	34	219	13.4
Limpopo	8	102	7.27
Mpumalanga	163	426	27.7
North West	108	279	27.9
Northern Cape	63	173	26.7
Western Cape	15	130	10.3
Total	1,107	2,683	29.2

2.3.7 Rabies in bats

Rabies cases in bats had only been recorded in the LP and KZN provinces over the 21-year period. For LP, 1.53% (*n = 5*) of all bat samples tested positive for rabies, while 4.00% (*n = 8*) all bat samples from KZN tested positive for rabies (**Table 2.8**).

Table 2.8: Suspect rabies samples collected from bat species across South Africa, 1998 - 2019

Province	Positive cases	Negative cases	% Positive
Eastern Cape	0	10	0.00
Free State	0	12	0.00
Gauteng	0	174	0.00
KwaZulu-Natal	8	192	4.00
Limpopo	5	322	1.53
Mpumalanga	0	44	0.00
North West	0	65	0.00
Northern Cape	0	3	0.00
Western Cape	0	3	0.00
Total	13	825	1.55

2.4 Discussion

By compiling and analysing South African surveillance data collected over a 21-year period, we gained valuable information on the epidemiological patterns of rabies across the country and were able to evaluate the relative impact of historical disease control initiatives. In addition, the epidemiological data used in this study not only allowed us to gain an improved understanding of rabies trends throughout the years and provinces, but also provided insight into the distribution of the species involved in rabies transmission across the geographical regions of the country as described.

Domestic dogs accounted for most of the positive rabies cases recorded in South Africa over the 21-year period. This observation was expected as dogs are well known to be the main reservoir species for rabies in most rabies-endemic countries – with dogs accounting for 99% of human rabies cases (Hampson *et al.*, 2015). Despite being endemic to the majority of the provinces, most of the positive canine rabies cases had originated from the eastern half of the country, with the highest number of cases recorded along the eastern seaboard (KZN and EC provinces) and in provinces adjacent to the neighbouring country of Mozambique (LP and MP provinces) (**Figure 2.5**). These observations follow a similar trend reported previously where the researchers found that the majority of canine rabies cases occurred in KZN, followed by the EC and MP provinces (Gummow, Roefs and de Klerk, 2010; Koeppel, van Schalkwyk and Thompson, 2021). This trend could, however, be explained by the fact that the human population densities were highest in those provinces (StatsSA, 2011 Census), resulting in proportionally high dog population densities in the province. These findings, as described by Cleaveland and co-workers (2014), would suggest that targeted disease intervention campaigns that focus specifically on the areas where human and dog population densities are

highest would be the most impactful approach to eliminating dog-mediated rabies using a resource-considerate approach in South Africa.

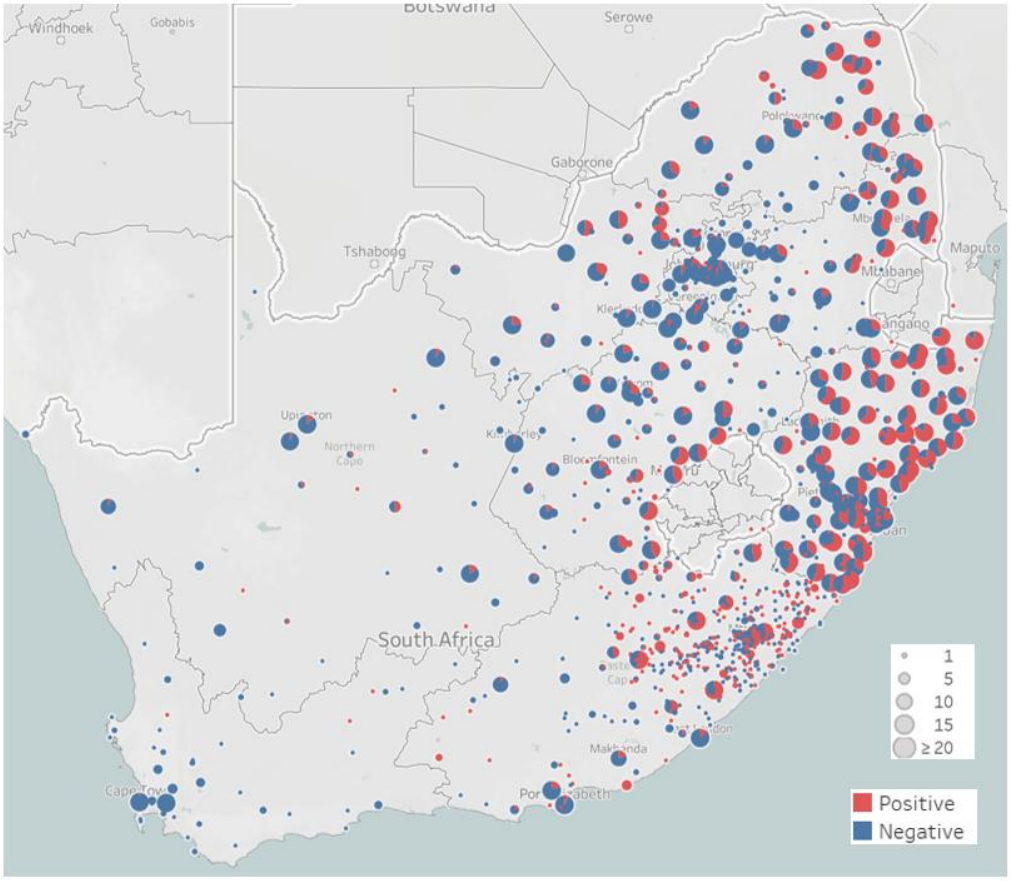

Figure 2.5: Geographic distribution of all rabies-positive and -negative cases from dogs in South Africa between 1998 and 2019.

Throughout the 21-year period, a relatively low number of rabies cases had originated from domestic cats in South Africa (*n = 332*). This was to be expected as findings reported from other countries had suggested that cats are a vector species and that rabies cases in cats thus occurred most often as a result of spill-over infections (Vaughn, 1975). In fact, at the time of writing there had not been any evidence to suggest that domestic cats were reservoir species that were capable of maintaining sustained rabies transmission in South Africa or elsewhere (Vaughn, 1975; Grobbelaar *et al.*, 2020).

Despite detecting rabies in cats in all the South African provinces between 1998 and 2019, the geographic distribution indicated that the majority of rabies cases in domestic cats were observed in the central parts of the country (**Figure 2.6**). Interestingly, a recent investigation into the human rabies cases associated with domestic cat exposures in South Africa found that rabies in domestic cats was linked to both the canid and mongoose variant of RABV (and

not rabies-related *Lyssavirus* species) in the country (Grobbelaar *et al.*, 2020). This would suggest that cats often get infected after an exposure to a rabid dog or mongoose – restricting the cat rabies cases to the parts of the country where those species get infected most often (e.g., the central parts of the country where mongoose rabies is most prevalent and the eastern seaboard where canine rabies is most prevalent) (**Figure 2.5**, **Figure 2.6** and **Figure 2.8**). These observations would suggest that the control and elimination of rabies in reservoir species whose geographic ranges overlap with domestic cats would prevent the occurrence of cat rabies cases.

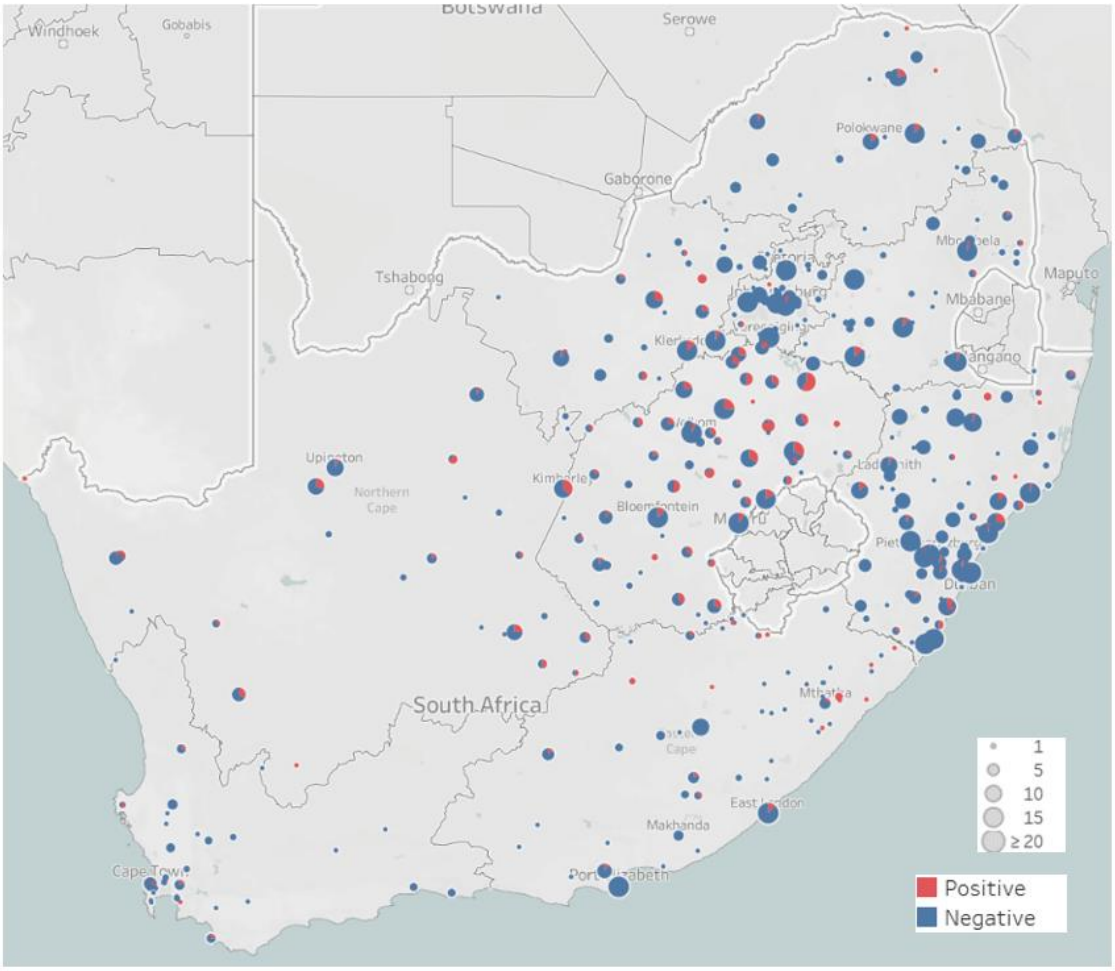

Figure 2.6: Geographic distribution of all rabies-positive and -negative cases from cats in South Africa between 1998 and 2019.

The findings of our investigation indicated that a significant number of rabies cases between 1998 and 2019 had originated from livestock (**Table 2.4**). Despite occurring across the country (**Figure 2.7**) the incidence of rabies cases in livestock largely coincided with those seen for domestic dogs – with the majority of cases occurring in the eastern half of South Africa. In contrast, only sporadic cases were reported from the western half of the country where canine

rabies cases occurred less often (**Figure 2.5** and **Figure 2.7**). This observation was in line with other published reports from Africa that found that the main source of rabies transmission to livestock was the domestic dog (Lembo *et al.*, 2008; Feng *et al.*, 2016; Balako *et al.*, 2018; Brookes *et al.*, 2019). In addition to the areas where livestock and dog rabies cases overlapped, some livestock cases did occur in parts of the country where dog rabies was not a common occurrence – albeit much less frequently (**Figure 2.5** and **Figure 2.7**). In those instances, the livestock rabies cases could be attributed to either the mongoose or canine variant transmitted by various species of wildlife (Vos *et al.*, 2014)(**Figure 2.8** and **Figure 2.10**). In support of this, the surveillance data used in this investigation showed that rabies cases in livestock followed a similar trend to those in mongooses – indicating a possible correlation in rabies persistence and outbreaks within these two species groups in areas that overlap geographically. These findings highlighted the importance of controlling rabies in all the animal populations residing in the areas in and around farms as a way to prevent spill-over infections to livestock.

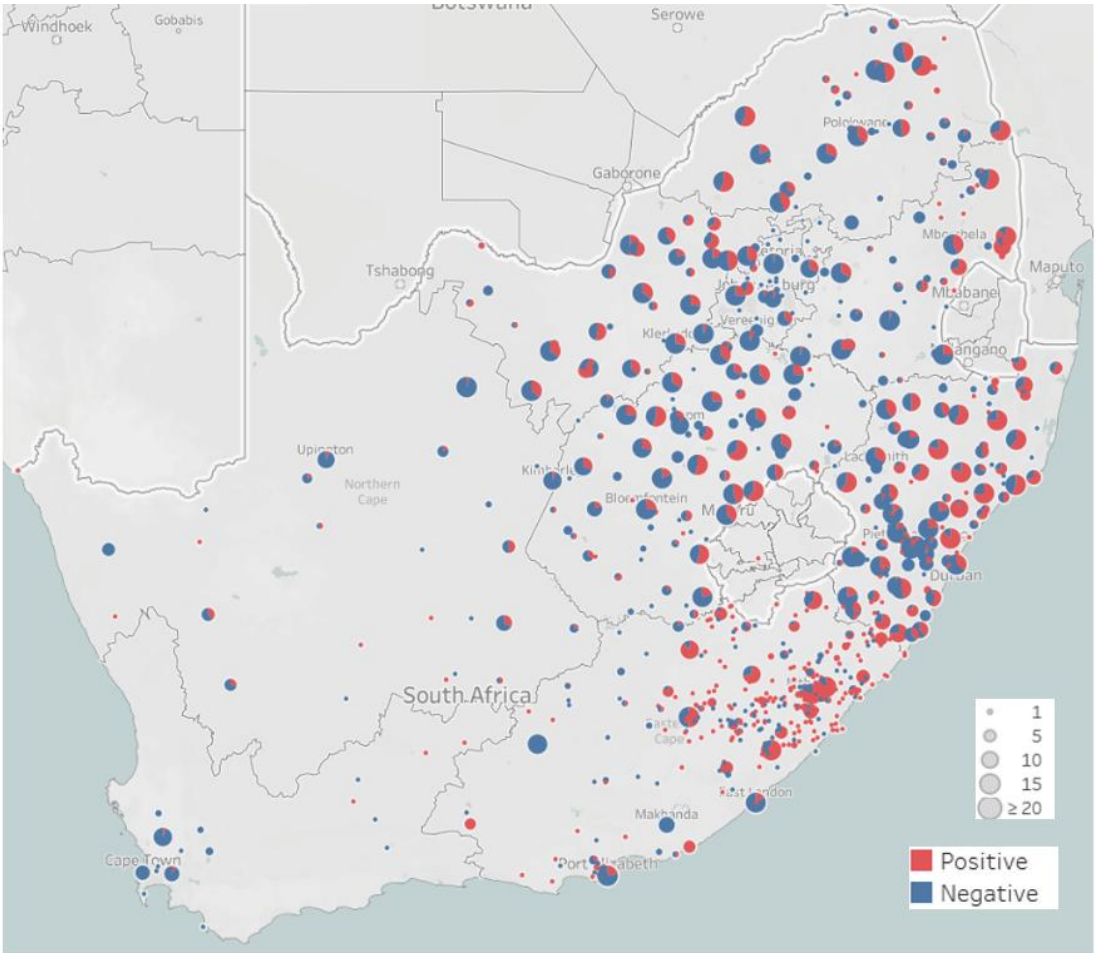

Figure 2.7: Geographic distribution of all rabies-positive and -negative cases from livestock in South Africa between 1998 and 2019.

While rabies cases in mongoose species were detected in all the South African provinces over the 21-year period, the majority of the cases had originated from the central plateau (*viz*. the FS, MP, and NW provinces) while also extending into the neighbouring GP, EC and NC provinces (**Table 2.8**). This observation was in-line with the existing knowledge that suggested that mongoose rabies cases were largely limited to the central parts of the country, with occasional cases occurring elsewhere in the country (Nel *et al.*, 2005; Davis *et al.*, 2007; Nel and Rupprecht, 2007a; Ngoepe, Sabeta and Nel, 2009; Van Sittert *et al.*, 2010; Van Zyl, Markotter and Nel, 2010). Geographic distributions for various mongoose species overlap, and as such, provides ample opportunity for the introduction of rabies into different mongoose populations (**Figure 2.9**). Historically, culling and gassing of dens were used as an effort to control the mongoose variant of RABV, however these measures proved to be ineffective (Swanepoel *et al.*, 1993). As such, future measures could include the use of ORVs to specifically target the mongoose variant of RABV in South Africa (Gilbert and Chipman, 2020).

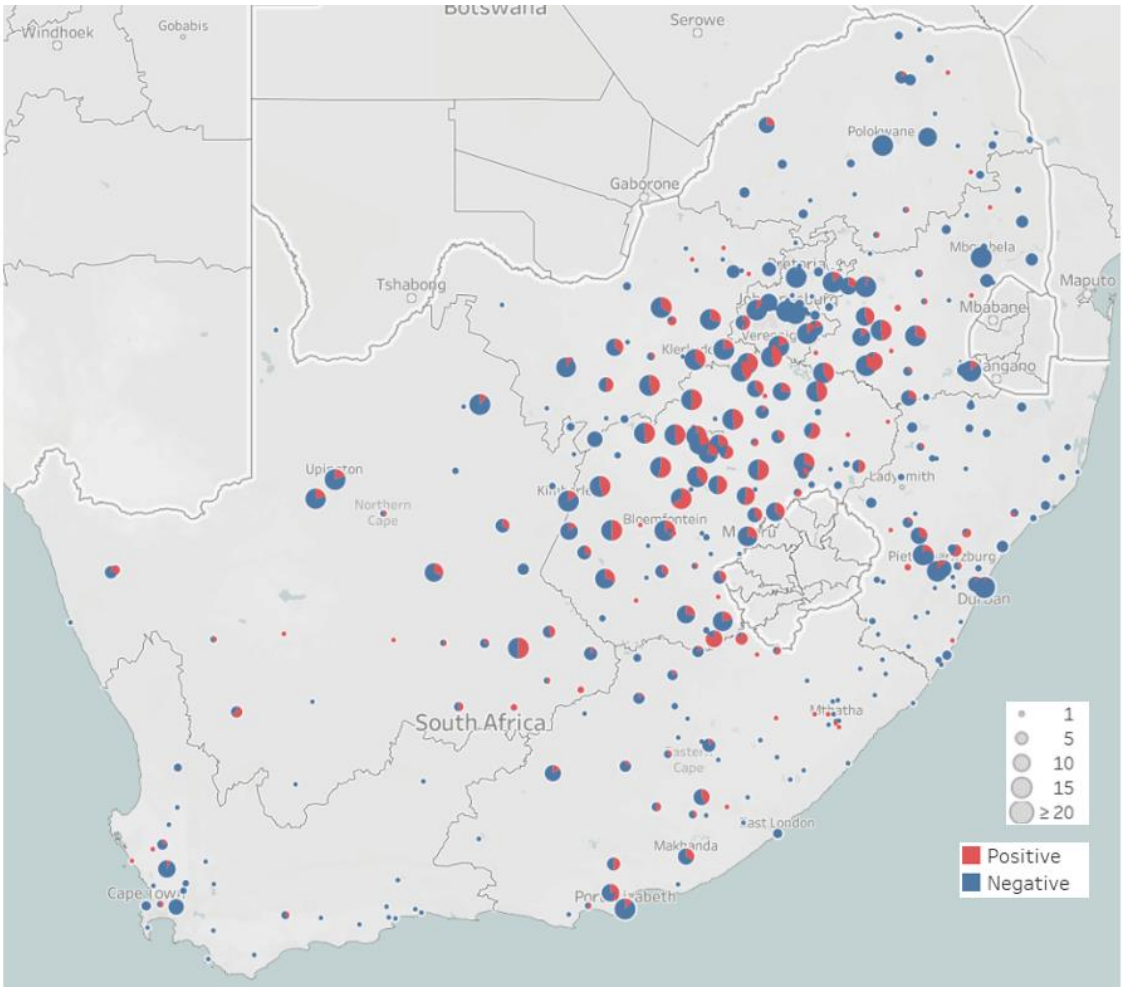

Figure 2.8: Geographic distribution of all rabies-positive and -negative cases from mongoose species in South Africa between 1998 and 2019.

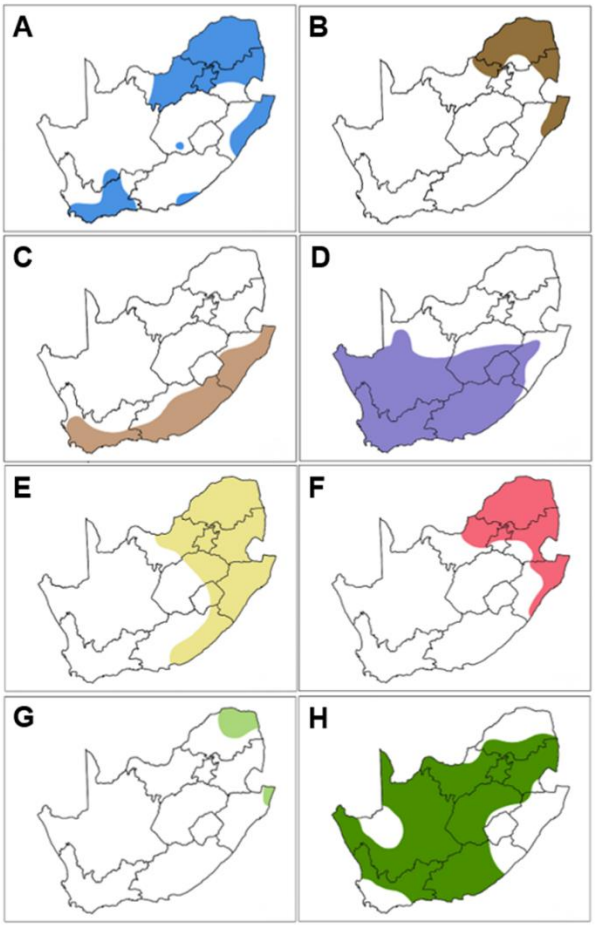

Figure 2.9: Species distribution maps for various mongoose species in South Africa associated with rabies transmission. (A: Water mongoose (*Atilax paludinosus*); B: Dwarf mongoose (*Helogale parvula*); C: Large grey mongoose (*Herpestes ichneumon*); D: Cape grey mongoose (*Herpestes pulverulentes*); E: White-tailed mongoose (*Ichneumia albicauda*); F: Banded mongoose (*Mungos mungo*); G: Selous mongoose (*Paracynictis selousi*); H: Suricate (*Suricata suricatta*)). The distribution for the yellow mongoose is shown in **Figure 2.2D**. Maps adapted using data generated by The Endangered Wildlife Trust (EWT, 2020).

Two rabies-related lyssavirus species associated with bat had been detected in South Africa to date, *viz*. LBV and DUVV (Coertse *et al.*, 2020). However, relatively few cases of rabies caused by these rabies-related lyssaviruses have been reported from South Africa (only 13 bat rabies cases for two provinces in the 21-year period). (**Table 2.8** and **Figure 2.10**). It should, however, be noted that these cases were detected via passive surveillance systems which is known to underestimate true prevalence. In contrast, active rabies surveillance programs specifically targeting bat species had found mounting antigenic and serological evidence that suggested that rabies-related lyssaviruses in bats are more common than what was initially realized (Picard-Meyer *et al.*, 2011; Coertse *et al.*, 2020, 2021).

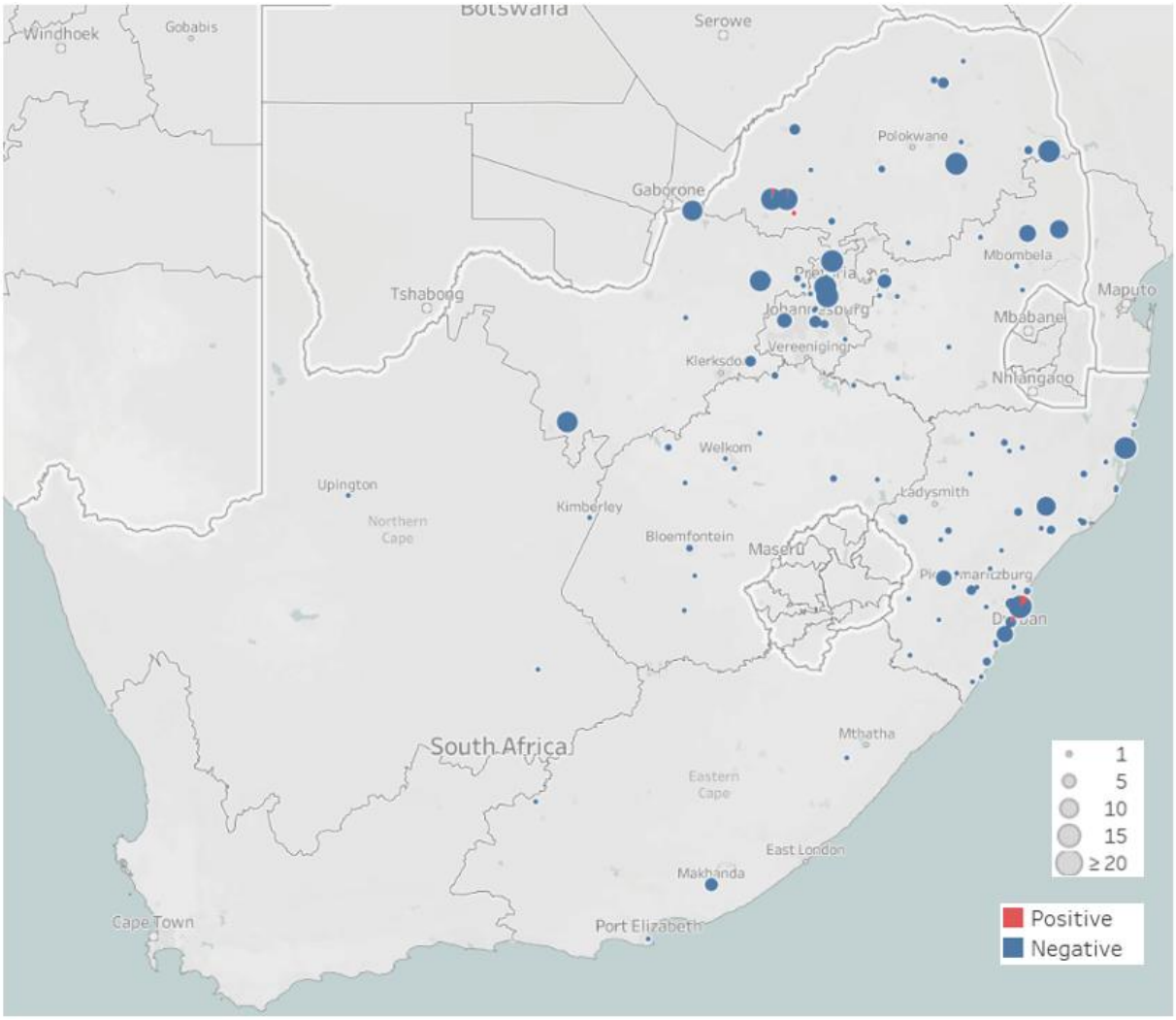

Figure 2.10: Geographic distribution of all rabies-positive and -negative cases from bats in South Africa between 1998 and 2019.

While positive rabies cases in sylvatic species were not as frequent as those in dogs (*n = 6,682*) and livestock (*n = 2,645*), sylvatic rabies cases were detected throughout the country over the 21-year period, with more cases being encountered in the northern regions where canine rabies cases were less frequently observed (**Figure 2.11** and **Figure 2.12**). Indeed, most of the rabies cases in jackals and bat-eared foxes were found in areas known to have lower human population densities and where farming activities predominated (**Figure 2.11**). While the rabies cases in other wildlife species (excluding jackals and bat-eared foxes) were evenly distributed throughout the country, the majority of the limited number of cases occurred where geographic ranges overlapped with jackals and bat-eared foxes (**Figure 2.12**).

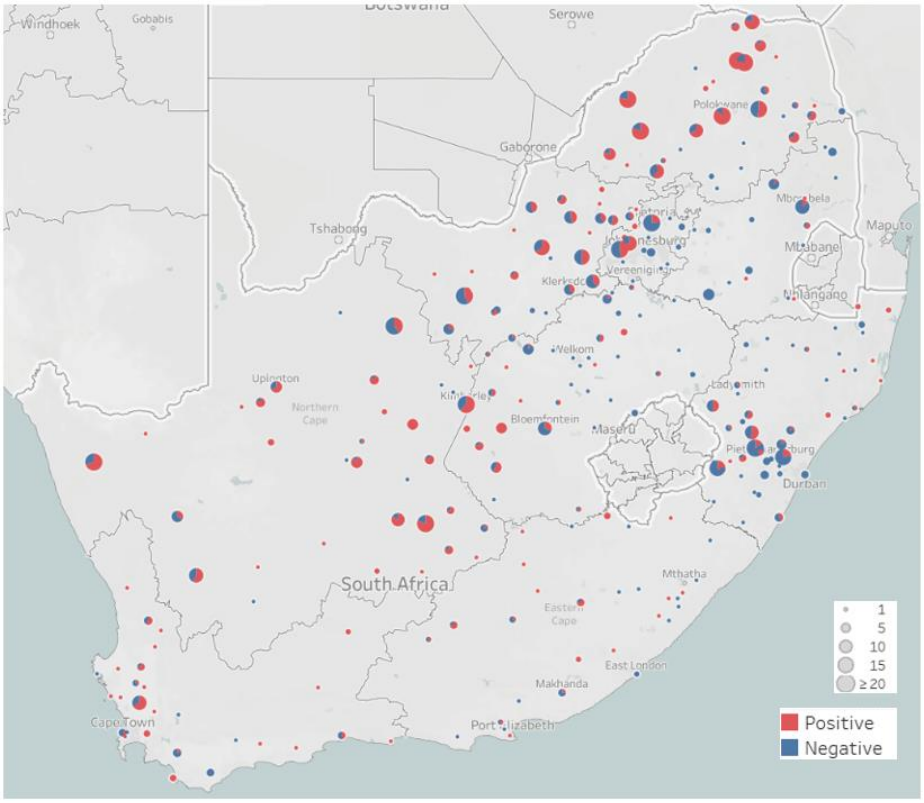

Figure 2.11: Geographic distribution of all rabies-positive and -negative cases from sylvatic reservoir species in South Africa between 1998 and 2019.

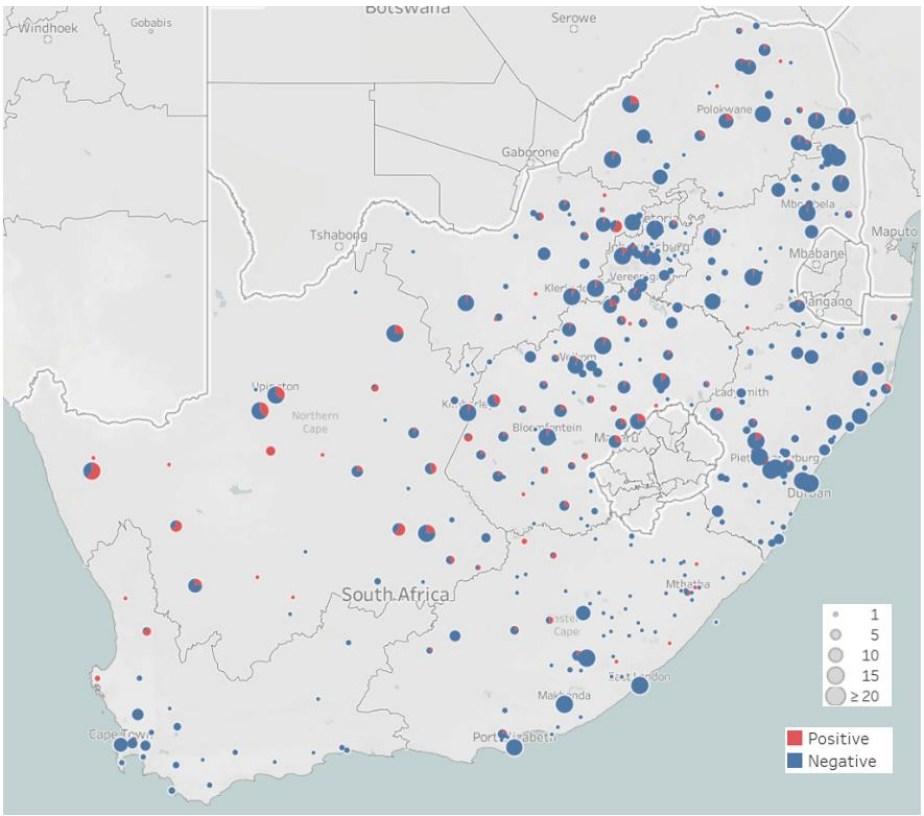

Figure 2.12: Geographic distribution of all rabies-positive and -negative cases from sylvatic vector species in South Africa between 1998 and 2019.

Considering that sylvatic rabies cases were present across the country – and had seemingly formed part of the epidemiology of rabies for the entire 21-year period – it is not surprising that some of the sylvatic rabies cycles had been subjected to molecular epidemiological investigations in the past. In evidence of the fact, sylvatic rabies cases in NC, WC, and KZN had been investigated thoroughly and their findings were broadly outlined below.

In the case of the NC and WC provinces, the role that bat-eared fox populations play in the transmission of rabies had been well documented (Thomson and Meredith, 1993; Sabeta *et al.*, 2007; Gummow *et al.*, 2010; Weyer *et al.*, 2011). In both provinces, bat-eared fox populations were able to maintain rabies in geographical areas where canine rabies cases were seldomly detected. In addition, molecular epidemiological investigations had indicated that the RABV sequences collected from rabies-positive bat-eared foxes were genetically distinct from those collected elsewhere in the country – suggesting that endemic cycles of sylvatic rabies had become established in the provinces (Thomson and Meredith, 1993; Sabeta *et al.*, 2007). In the case of KZN, a province where dogs had historically been the main reservoir species for rabies, an outbreak of sylvatic rabies was recorded in 2012. The outbreak was investigated and found to be as a result of a spill-over event of canine rabies into the black-backed jackal populations, which resulted in the establishment of an independent sylvatic rabies cycle (KwaZulu-Natal Department of the Agriculture Rural Development, 2015).

Although sylvatic rabies cases had been reported in the remaining provinces of South Africa provinces, the surveillance data would suggest that those from the FS, MP, GP and the EC provinces were most likely due to spill-over infections from rabid dogs. In support of this, the surveillance data indicated that most of those sporadic sylvatic cases occurred in areas where high numbers of canine rabies cases were found (**Figure 2.5, Figure 2.11** and **Figure 2.12**). This would suggest that the cases occurred as a result of chance encounters between rabid dogs and sylvatic species, and not necessarily as a result of maintained transmission amongst the sylvatic species (Ngoepe *et al*., 2009; Mkhize *et al.*, 2010; Van Sittert *et al.*, 2010; Sabeta *et al.*, 2013).

In two of the provinces (*viz*. NW and LP provinces), however, the surveillance data alluded to the presence of persistent transmission of rabies within the sylvatic populations in areas that were geographically distinct from where the canine rabies cases occurred. In the NW, the surveillance data indicated that positively reported rabies cases in sylvatic species could be found throughout the province (**Figure 2.11** and **Figure 2.12**), while canine rabies cases were predominantly found in the eastern half of the province (**Figure 2.5**). Surveillance data for LP showed that sylvatic rabies cases could also be found throughout the province, with most cases being found in the central strip and western half of the province (**Figure 2.11** and **Figure**

2.12). Meanwhile, canine rabies cases were mainly found in the eastern half of the province (**Figure 2.5**).

In light of these findings – and the geographic distribution of the various species throughout the two provinces – we speculated that rabies was being maintained within the sylvatic populations of the NW and LP provinces and thus undertook molecular epidemiological analyses to better understand the relationship of rabies endemic cycles circulating in canine and sylvatic species in the two provinces (**Chapter III** and **IV**).

Chapter III

Molecular epidemiology of domestic and sylvatic rabies in the North West province of South Africa

3.1 Introduction

The NW province is situated in the northern regions of South Africa and shares a political border with four South African provinces (*viz*. the NC, FS, GP and LP provinces) and the neighbouring country of Botswana (**Figure 3.1**). While the first case of canine-mediated rabies in NW was only identified in 1980, anecdotal evidence suggests that rabies was introduced into the province in the 1950s from the neighbouring LP Province (formerly part of the Transvaal province) where it has remained endemic since.

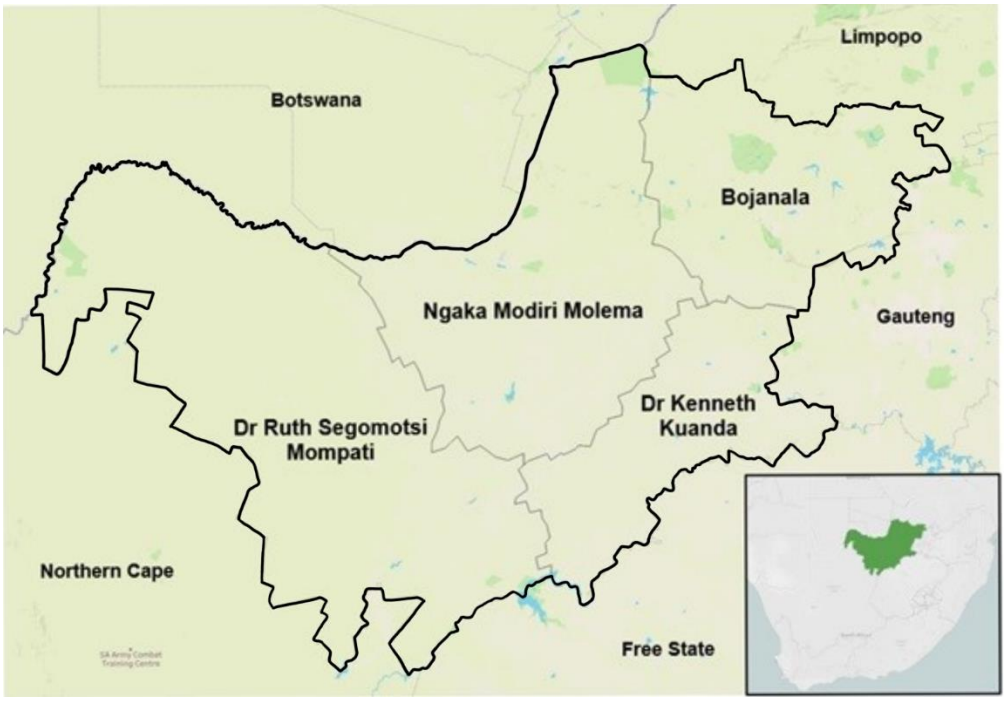

Figure 3.1: Map showing the districts of the NW province in SA (Municipalities of South Africa, 2021)

As discussed in Chapter II, suspected rabies samples originating from various animal species across the province are sent for diagnostic confirmation each year, with specimens collected from suspect rabid dogs, livestock and sylvatic species being submitted most frequently (**Table 2.2**, **2.4**, **2.5**, and **2.6**). Despite having a diverse dataset of surveillance data indicating that canine rabies cases were predominately found in the eastern half of the province, while livestock and sylvatic rabies cases were found throughout the province, the epidemiological information on the prevailing endemic cycles and disease transmission within this province had been very limited to date.

In an effort to supplement the limited empirical surveillance data – and gain an improved understanding of the prevailing epidemiological landscape – molecular epidemiological investigations focussing on other South African provinces had included sequences originating from the NW province in the past. In one such study, focussing primarily on the molecular epidemiology of rabies in LP, RABV isolates from domestic dogs and black-backed jackals

originating from the NW and LP provinces were found to be genetically similar (Zulu *et al.*, 2009). This study was, however, largely limited to samples that had been obtained from the LP province and did not include a diverse set of sequences from the NW province. Another such study investigated an outbreak of canine rabies in the GP province in 2010 (Sabeta *et al.*, 2013). While rabies cases have been reported sporadically throughout the years in the GP province, the outbreak lead to the spread of the disease through the western half of the province (Sabeta *et al.*, 2013). Although a RABV sequence originating from a mongoose species collected from the NW province was genetically similar to a RABV sequences collected from a mongoose species originating in GP, no other RABV sequences originating from the NW were included in the study. As such, no firm conclusions could be drawn with regards to the impact of rabies in NW in relation to the rabies outbreak in GP (Sabeta *et al.*, 2013).

As no previous molecular epidemiological studies have focused on the NW province, the relationship between domestic and wildlife rabies cycles in the NW are not particularly well understood. Our aim therefore was to improve our understanding of the molecular epidemiology of rabies in NW, and to investigate the genetic relationship between canine and sylvatic rabies within the NW. In addition, we investigated the genetic homology between RABV sequences collected from the NW and GP provinces in an effort to determine whether the outbreak of rabies in GP could be epidemiologically linked to rabies in NW.

3.2 Materials and Methods

3.2.1. Sample cohort used in study

A cohort of contemporary rabies-positive samples from various regions throughout the NW and GP provinces (routine surveillance collection 2017-2019) were selected based on the species and the geographical distribution throughout the province (*n = 53*).

The geographic distribution for all sequences included in the phylogenetic analyses were visualised using the Tableau software package (version 2020.4.1, Seattle, USA) and relied on the use of geographic coordinates associated with each sample.

3.2.2. RNA extraction

The total RNA was extracted from each of the 53 samples by using the Zymo Direct-zol RNA MiniPrep Plus kit (Zymo Research, USA) according to the manufacturer's instructions.

Briefly, 50 – 100 mg of brain material was homogenised in 600 µl TRIzol™ Reagent (Thermo Fisher Scientific, USA). The homogenised brain samples were centrifuged for 30 seconds at 10,000 g to remove particulate matter, and the supernatant was transferred to a sterile 1.5 ml

microcentrifuge tube (Thermo Fisher Scientific). An equal volume of 100% ethanol (Merck Chemicals, South Africa) was added, and the solution was mixed thoroughly. The liquid phase was transferred to a Zymo-Spin™ IIICG Column in a collection tube and centrifuged for 30 seconds at 10,000 g. To prepare the column for RNA extraction, 400 µl of the Direct-zol™ RNA PreWash was added to the column and centrifuged at 10,000 g for 30 seconds. The flow-through was discarded before repeating the pre-wash step a second time. The RNA in the spin column was washed by adding 700 µl RNA Wash Buffer to the column before centrifuging for two minutes at 10,000 g to ensure the complete removal of the Wash Buffer. The Zymo-Spin™ IIICG Column was transferred to a sterile 1.5 ml microcentrifuge tube (Thermo Fisher Scientific) and the RNA eluted by adding 100 µl of RNase-free water directly to the column matrix before centrifugation for 30 seconds at 10,000 g. The extracted total RNA was stored at -80°C until use.

3.2.3. Reverse transcription

The reverse transcription of the isolated total RNA was done using an established protocol (Markotter *et al.*, 2006). For all reactions, a positive control (containing RABV RNA of known concentration and origin) and negative control (using nuclease-free water instead of RABV RNA) were included.

For the reverse transcription reaction, 10 pmol forward primer (001lys) (**Table 3.1**) was added to 5 µl of total RNA in a sterile 0.2 ml microcentrifuge tube (Merck). The tube was heated to 94°C for one minute, after which the tube was immediately placed on ice for five minutes. The reverse transcription of each sample was subsequently done by adding 7.3 µl of nuclease-free water (Promega, United States), 4.5 µl of SuperScript reaction buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$) (Invitrogen, USA), 2.2 µl dNTP mix (10mM) (Promega), 0.4 µl of SuperScript reverse transcriptase (200 U/µl) (Invitrogen) and 0.4 µl Ribolock® Ribonuclease Inhibitor (40 U/µl) (Thermo Fisher Scientific) to the reaction mix before incubating the tubes 42°C for 90 minutes. All enzymes were subsequently inactivated by heating the tube to 70°C for 15 minutes.

Table 3.1: Oligonucleotide primers for PCR amplification of the glycoprotein gene and the adjacent G-L intergenic region, and the partial N gene of the RABV genome

Primer	Primer sequence*	Used for	Position on genome[#]
001lys	5'- ACGCTTAACGAMAAA -3'	cDNA synthesis; PCR and forward sequencing of the partial N gene sequences in combination with the 550B primer.	16 – 30
G+	5'-GACTTGGGTCTCCCAACTGGGG -3'	PCR and forward sequencing of G-L intergenic region in combination with the L- primer.	4665 – 4687
L-	5'- CAAAGGAGAGTTGAGATTGTAGTC -3'	PCR and reverse sequencing of G-L intergenic region in combination with the G+ primer.	5543 – 5566
550B	5'- GTRCTCCARTTAGCRCACAT -3'	PCR and reverse sequencing of the partial N gene sequences in combination with the 001lys primer.	647 - 666

* "M" IUPAC code represents either an A or C nucleotide
[#] Nucleotide positions numbered according to the Pasteur virus strain (GenBank accession number: M13215)

3.2.4. Polymerase chain reaction amplification of the glycoprotein gene and the adjacent G-L intergenic region

After reverse transcription of the total RNA for each sample, the G-L intergenic region of the RABV genome was amplified using a specific primer set (**Table 3.1**) and established protocol (Sacramento *et al.*, 1991; von Teichman *et al.*, 1995).

For each PCR reaction, 10 µl of cDNA was mixed with 0.50 µl of the forward primer (G+, 10 pmol), 0.65 µl of the reverse primer (L-, 10 pmol), 14 µl nuclease-free water, and 25 µl of the DreamTaq master mix (DreamTaq DNA polymerase, 2x DreamTaq buffer, dATP, dCTP, dGTP, dTTP (4 mM each) and 4 mM $MgCl_2$) (Thermo Fisher Scientific) before being subjected to nucleic acid amplification according to the following cycling conditions: one cycle at 94°C for two minutes, 30 cycles at 94°C for 50 seconds, 42°C for 90 seconds and 72°C for two minutes, and a final extension cycle at 72°C for seven minutes. The positive and negative controls generated during reverse transcription were included to confirm the fidelity of the PCR reaction.

3.2.5. Polymerase chain reaction amplification of the partial nucleoprotein gene

After reverse transcription of the total RNA for each sample, the partial N gene of the RABV genome was amplified using a specific primer set (**Table 3.1**) and established protocol (Markotter *et al.*, 2006).

For each PCR reaction, 10 µl of cDNA was mixed with 0.5 µl of the forward primer (001lys, 10 pmol), 0.65 µl of the reverse primer (550B, 10 pmol), 14 µl nuclease-free water, and 25 µl of the DreamTaq master mix (DreamTaq DNA polymerase, 2x DreamTaq buffer, dATP, dCTP, dGTP, dTTP (4 mM each) and 4 mM $MgCl_2$) (Thermo Fisher Scientific) before being subjected to the following cycling conditions: one cycle at 94°C for one minute, 40 cycles at 94°C for 30 seconds, 37°C for 30 seconds and 72°C for 90 seconds, and a final extension cycle at 72°C for seven minutes. The positive and negative controls generated during reverse transcription were included to confirm the fidelity of the PCR reaction.

3.2.6. Modified polymerase chain reaction amplification of the partial nucleoprotein gene

The partial N gene PCR protocol described above (**Section 3.2.6**) failed to amplify nucleic acid for some of the samples (*n = 8*) and resulted in insufficient levels of amplified nucleic acid for other samples (*n = 10*).

3.2.6.1. Taguchi optimization of the partial nucleoprotein gene polymerase chain reaction

The partial N gene PCR protocol (Section 3.2.6) was optimised using the Taguchi protocol for PCR optimisation (**Table 3.2**) (Cobb and Clarkson, 1994). Briefly, a set of preselected variables (*viz*. PCR primer annealing temperature, addition of $MgCl_2$ to the PCR master mix, starting volume of cDNA included in the PCR reaction and the RNA concentration used for cDNA synthesis) were tested in nine separate reactions to determine optimal amplification conditions (**Table 3.2**). Thereafter, the ideal modified partial N gene PCR protocol was selected by band intensity after gel electrophoresis (**Figure A1; Appendix materials).**

Table 3.2: Variables associated with the Taguchi optimisation of the partial nucleoprotein gene polymerase chain reaction

		Levels		
	Variables	**A**	**B**	**C**
[1]	**Primer annealing temp (PCR reaction)**	30°C	37°C	45°C
[2]	**RNA concentration (reverse transcription reaction)**	1:15	1:10	1:5
[3]	**$MgCl_2$ (PCR reaction)**	4.0 mM (0 µl)	3.5 mM (7 µl)	6 mM (12 µl)
[4]	**cDNA volume (PCR reaction)**	5 µl	15 µl	20 µl

Variables (→)	**[1]**	**[2]**	**[3]**	**[4]**
Reaction number (↓)				
1	A	A	A	A
2	A	B	B	B
3	A	C	C	C
4	B	A	B	C
5	B	B	C	A
6	B	C	A	B
7	C	A	C	B
8	C	B	A	C
9	C	C	B	A

Each column represents a variable that was changed while each row represents a separate reaction

3.2.6.2. Modified partial nucleoprotein gene polymerase chain reaction protocol

The revised partial N gene PCR protocol relied on a modification to both the reverse transcription reaction and the N gene PCR reaction.

For the reverse transcription of the cDNA, the published protocol (Markotter *et al.*, 2006) was adapted by diluting the initial RNA concentration to a final 1:10 concentration with nuclease-free water (Promega) for cDNA synthesis. Thereafter, the revised partial N gene PCR reaction was set up as follows: 5 µl of cDNA was mixed with 0.5 µl of the forward primer (001lys, 10 pmol) (**Table 3.1**), 0.65 µl of the reverse primer (550B, 10 pmol) (**Table 3.1**), 9.3 µl nuclease-free water (Promega), and 25 µl of the DreamTaq (Thermo Fisher Scientific) master mix (DreamTaq DNA polymerase, 2x DreamTaq buffer, dATP, dCTP, dGTP, dTTP (4 mM each) and 4 mM $MgCl_2$) before being subjected to nucleic acid amplification. The amplification using these new variables was done using the following cycling conditions: one cycle at 94°C for one minute, 40 cycles at 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 90 seconds, and a final extension cycle at 72°C for seven minutes.

3.2.7. Agarose gel electrophoresis and excision of the amplified nucleic acid

A 45 µl aliquot of each of the PCR-positive amplicons (partial N gene, *n = 53*; G-L intergenic region, *n = 53*) was added to 9 µl of loading dye (40% sucrose and 0.25% bromophenol blue) and electrophoresed on a standard 1% agarose gel. The electrophoresed products were observed under UV light and excised from the agarose gel using a sterile scalpel blade before being transferred to a sterile 1.5 ml microcentrifuge tube (Merck).

3.2.8. PCR clean-up

The PCR clean-up procedure for all the amplified nucleic acid products (partial N gene, *n = 53*; G-L intergenic region, *n = 53*) was done using the Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions.

Three volumes of agarose dissolving buffer (ADB) solution were added to each excised agarose gel slice and the tube incubated at 54°C for ten minutes until the gel slice had completely dissolved. The melted agarose solution was transferred to a clean Zymo-Spin™ Column in a collection tube and centrifuged at 10,000 g for 60 seconds. The flow-through was discarded and 200 µl of DNA wash buffer was added to the column. The tubes were centrifuged at 10,000 g for 30 seconds, before the flow-through was discarded and the wash step repeated. To elute the DNA from the column, the Zymo-Spin™ Column was transferred to a clean 1.5 ml microcentrifuge tube (Merck) and 30 µl of DNA elution buffer was added directly to the column matrix. This column was centrifuged at 10,000 g for 60 seconds and the eluted DNA used for the subsequent sequencing reactions.

3.2.9 Sanger sequencing

The forward (5'-3') and reverse (3'-5') strands of all the amplified nucleic acid was subjected to Sanger sequencing and precipitated reaction at Inqaba Biotec™ (Pretoria, South Africa) using the ABI Prism 3500XL Genetic Analyzer (ThermoFisher). The final consensus sequences (*n = 53*) were trimmed to 405 nucleotides (nt) for the partial N gene and 592 nt for the G-L intergenic region using the CLC Main Workbench software (version 20.0.1). The trimmed consensus sequences, representing the G-L intergenic region and partial N gene of the RABV genome for samples from the NW (*n = 51*) and GP (n = 2) provinces, was submitted to the NCBI GenBank and allocated unique accession numbers (G-L: MW343859 – MW343912; N: MW343969 – MW344022; **Table A2, A3**).

3.2.10 Phylogenetic analysis

The phylogenetic analysis included the 53 RABV sequences from the NW and GP provinces generated in this study as well as published G-L intergenic region (*n = 20*) and partial nucleoprotein gene (*n = 23*) sequences that had been obtained from South Africa (NW, GP, LP, FS, and KZN provinces) and the neighbouring countries of Zimbabwe and Botswana.

Due to the low number of published partial N gene sequences from the southern African region it was not possible to undertake the phylogenetic analyses with the exact same panel of RABVs for both the G-L intergenic and partial N gene regions. This was because, while the N gene and G-L intergenic region sequences had been generated for some RABVs, only the N gene or the G-L intergenic region sequences had been generated for others. To overcome this limitation, a highly similar panel of RABV G-L intergenic region sequences was created by including published RABVs where the N gene and G-L intergenic region sequences were available (*n = 17*) and supplementing the panel with published RABV G-L intergenic region sequences from samples that had been collected from the same geographical area (at a similar time point) as the samples which were used to generate the partial N gene sequences (*n = 5*). This enabled a comparative panel to be created under the assumption that the samples would have been collected from the same endemic cycles and would thus be genetically similar. The only exception to this approach was for the RABV sequence from Botswana as no G-L intergenic region sequences were available from the country.

As such, two separate alignments were created in this investigation – one for the partial N gene (*n = 77*) and one for the G-L intergenic region (*n = 76*) sequences. The sequences for both datasets were aligned using the ClustalW subroutine of the MEGA X software package, and the best-fitting DNA substitution models (partial N gene: TrN+G; G-L intergenic region:

TIM1+G) were determined using the JModel software package (version 2.1.10) using the Akaike's information criterion (AIC).

The final phylogenetic analysis for both gene regions was undertaken using a Bayesian Markov Chain Monte Carlo (MCMC) method in the BEAST software package (version 2.6.0) (Drummond *et al*., 2012). The phylogenetic analysis relied on three independent Markov chains sampled for 10 million states and a sampling frequency of 10,000 was combined after discarding at least a 10 per cent burn. The posterior distributions were subsequently inspected using the Tracer software (version 1.7.1) to ensure adequate mixing and convergence before the associated statistics were summarised as a maximum clade credibility tree and visualised using the FigTree software (version 1.4.4).

3.3 Results

3.3.1. Sample cohort for specimens included in the molecular epidemiological analysis

A cohort of 53 rabies-positive brain specimens were selected for inclusion in the molecular epidemiological investigation (**Figure 3.2**; **Table A2, A3**). The animal species selected for inclusion in this investigation had been collected during routine surveillance and included the following: canine (*n = 12*); bovine (*n = 21*); black-backed jackal (*n = 9*); bat-eared fox (*n = 1*); ovine (*n = 2*); caprine (*n = 3*); unknown jackal species (*n = 3*); aardwolf (*n = 1*); and genet (*n = 1*) (**Table A2, A3; Appendix materials**).

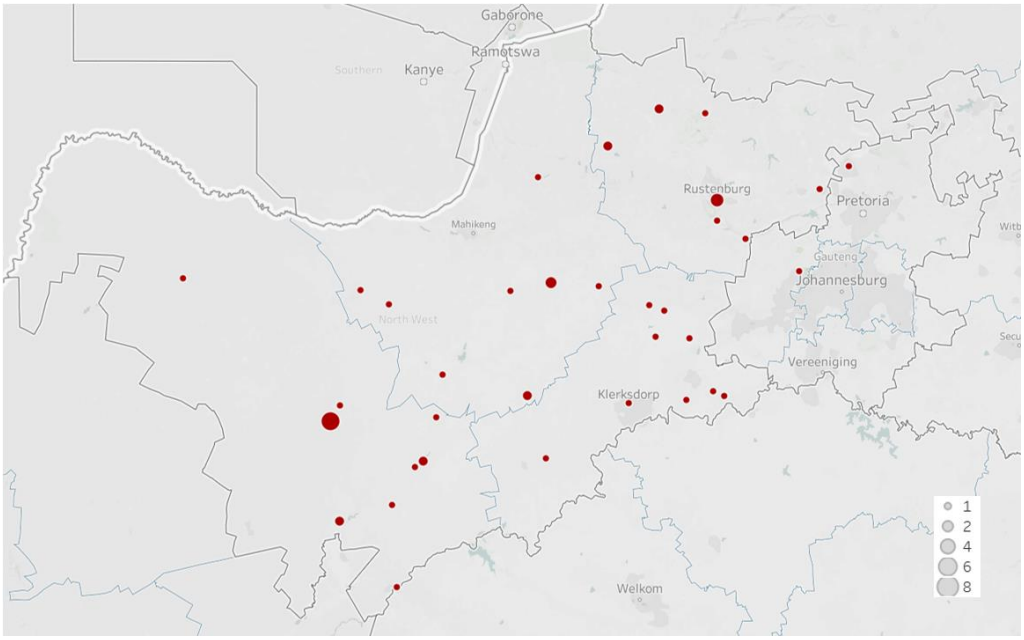

Figure 3.2: Geographic locations of the contemporary rabies-positive samples included in this study from the NW (*n = 51*) and GP (*n = 2*) provinces. The geographic distribution for all sequences included in the phylogenetic analyses were visualised using the Tableau software package (version 2020.4.1, Seattle, USA).

3.3.2 Phylogenetic analysis of the partial N gene for RABV sequences derived from the North West province of South Africa

The partial N gene sequences included in this study disaggregated into four separate clades (Clade A – D) with each clade supported by high posterior probabilities (**Figure 3.3**). Clade A consisted of RABV sequences that originated from within the NW province. Clade B consisted of RABV sequences from the NW, GP, KZN and LP provinces of South Africa. Clade C consisted of RABV sequences from Botswana, Zimbabwe, and the NW province of South Africa, while clade D consisted of RABV sequences from the NW and FS provinces in South Africa (**Figure 3.3**).

Clade A consisted of 30 RABV sequences collected from canine (*n = 3*), bovine (*n = 15*), black-backed jackal (*n = 7*), ovine (*n = 1*), caprine (*n = 1*), bat-eared fox (*n = 2*), and an unspecified jackal species (*n = 1*). The RABV sequences forming part of Clade A were predominantly geographically limited to the western parts of the NW province (**Figure 3.4**).

Clade B consisted of 39 RABV sequences collected from canine (*n = 17*), bovine (*n = 5*), black-backed jackal (*n = 10*), caprine (*n = 2*), ovine (*n = 1*), aardwolf (*n = 1*), African wild dog (*n = 1*), and unspecified jackal species (*n = 2*). The RABV sequences from the NW province in this clade (Clade B) were geographically limited to the eastern parts of the province, suggesting that the RABV sequences originated from the eastern parts of the NW province shared genetic relatedness with those collected from both the GP, LP, and KZN provinces of South Africa, as well as the neighbouring country of Mozambique (**Figure 3.4**).

Interestingly, Clade C consisted of previously published RABV sequences collected from African civets in Zimbabwe (*n = 2*), which clustered with RABV sequences collected from a genet in South Africa (*n = 1*) and a jackal in Botswana (*n = 1*). The clustering pattern observed in this clade indicated genetic homology between RABV sequences, suggesting that long-range, transboundary movement of infected animals between Zimbabwe, Botswana, and the northern parts of the NW province of South Africa had taken place (**Figure 3.4**).

The last clade, Clade D, consisted of RABV sequences collected from mongooses from the FS (*n = 1*) and NW provinces (*n = 1*), and bovine samples from the NW province (*n = 2*). The RABV sequences in this clade all belonged to the mongoose variant of the RABV, suggesting that the mongoose variant of the RABV extended from the FS province into the central parts of the NW province (**Figure 3.3** and **Figure 3.4**).

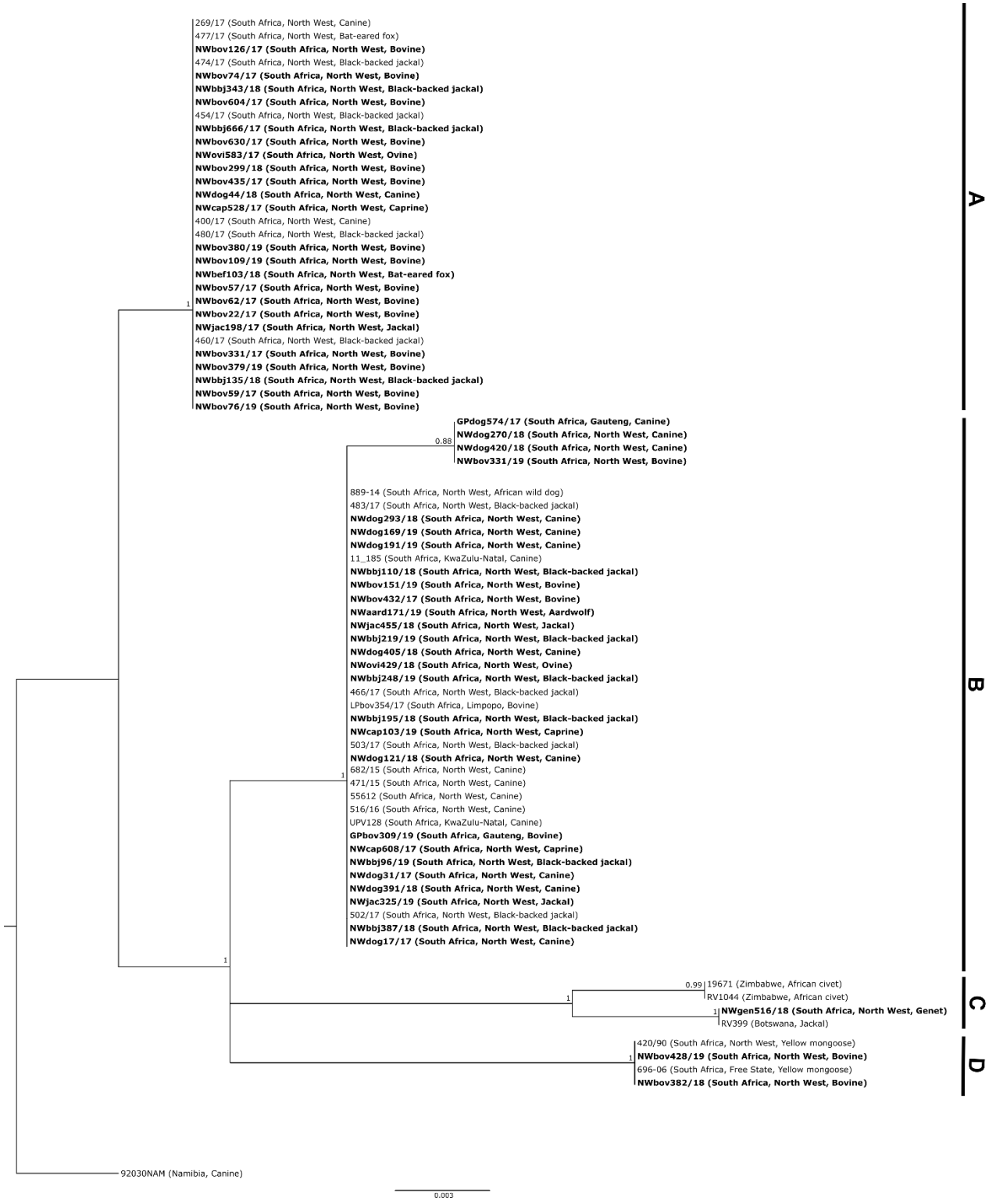

Figure 3.3: Maximum clade credibility tree for partial N gene sequences derived from South Africa, Zimbabwe, and Botswana (**Table 3.3**, **Table A2**). The horizontal branch lengths are proportional to the homology between sequences within and between groups and all branches with a posterior probability of 0.75 or less were collapsed. A canine sequence from Namibia (92030NAM) was used to root the tree. The new sequences generated in this study are shown in bold (**Table 3.3**, **Table A2**). All sequences in Clade A and Clade B belong to the Africa 1-b lineage, while the sequences in Clade C and Clade D belong to the Africa 3 lineage (**Table 3.3**).

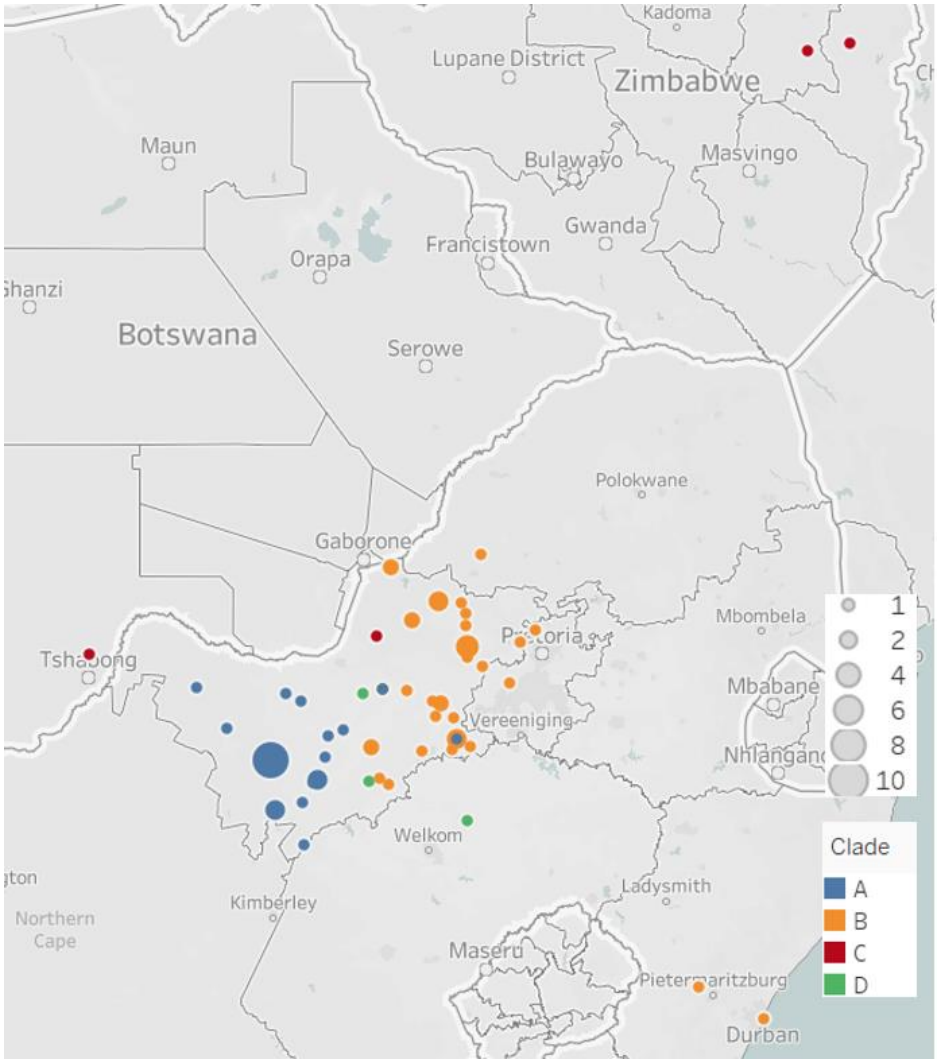

Figure 3.4: Geographic distribution for sequences from each clade as seen in the phylogenetic analysis for the partial N gene sequences.

Table 3.3: Sample cohort for all RABV sequences included in the phylogenetic analyses for samples from rabies-positive animals included in this study

Year Sampled	Sample Number	Species	Country	Province/ Region	Latitude	Longitude	Africa Lineage
1989	399	Jackal	Botswana	Tshabong	-25.754	22.41838	3
1990	m466	Yellow mongoose	South Africa	Free State	-27.374	26.61996	3
1990	420/90	Yellow mongoose	South Africa	North West	-27.1974	25.98311	3
1991	19671	African civet	Zimbabwe	Rusape	-18.5279	32.12843	3
1994	22574	African civet	Zimbabwe	Wedza	-18.6173	31.5736	3
2003	KZNdg03.453	Canine	South Africa	KwaZulu-Natal	-29.8579	31.0292	1-b
2006	696/06	Yellow mongoose	South Africa	Free State	-27.6504	27.23488	3
2008	UPV128	Canine	South Africa	KwaZulu-Natal	-29.8579	31.0292	1-b

2011	11_185	Canine	South Africa	KwaZulu-Natal	-29.5093	30.19838	1-b
2012	555/12	Hyena	South Africa	North West	-25.2775	27.21605	1-b
2012	556/12	Canine	South Africa	North West	-25.2775	27.21605	1-b
2012	566/12	Black-backed jackal	South Africa	North West	-25.2775	27.21605	1-b
2014	889/14	African wild dog	South Africa	North West	-24.7435	26.25732	1-b
2015	KZNbov15/261	Bovine	South Africa	KwaZulu-Natal			1-b
2015	113/15	Hyena	South Africa	North West	-24.7435	26.25732	1-b
2015	471/15	Canine	South Africa	North West	-27.2371	26.23514	1-b
2015	682/15	Canine	South Africa	North West	-24.7435	26.25732	1-b
2016	516/16	Canine	South Africa	North West	-25.4261	27.2243	1-b
2016	635/16	Canine	South Africa	North West	-25.8026	27.87506	1-b
2017	NWdog17/17	Canine	South Africa	North West	-25.7905	27.2421	1-b
2017	NWbov22/17	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2017	NWdog31/17	Canine	South Africa	North West	-25.1609	27.16296	1-b
2017	NWbov57/17	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2017	NWbov59/17	Bovine	South Africa	North West	-27.1887	25.32931	1-b
2017	NWbov62/17	Bovine	South Africa	North West	-26.8648	24.79046	1-b
2017	NWbov74/17	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2017	NWbov126/17	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2017	NWjac198/17	Jackal	South Africa	North West	-26.6864	25.45907	1-b
2017	NWbov331/17	Bovine	South Africa	North West	-27.1887	25.32931	1-b
2017	NWbov432/17	Bovine	South Africa	North West	-26.1739	26.46947	1-b
2017	NWbov435/17	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2017	NWcap528/17	Caprine	South Africa	North West	-27.5311	24.78659	1-b
2017	NWovi583/17	Ovine	South Africa	North West	-27.5311	24.78659	1-b
2017	NWbov604/17	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2017	NWcap608/17	Caprine	South Africa	North West	-25.1334	26.86546	1-b
2017	NWbov630/17	Bovine	South Africa	North West	-26.125	23.7725	1-b
2017	NWbbj666/17	Black-backed jackal	South Africa	North West	-26.2802	25.10966	1-b
2017	GPdog574/17	Canine	South Africa	Gauteng	-25.4729	28.09919	1-b
2017	269/17	Canine	South Africa	North West	-26.9566	24.7284	1-b
2017	400/17	Canine	South Africa	North West	-26.9566	24.7284	1-b
2017	454/17	Black-backed jackal	South Africa	North West	-26.7167	27.1	1-b

2017	460/17	Black-backed jackal	South Africa	North West	-26.5961	24.17612	1-b
2017	474/17	Black-backed jackal	South Africa	North West	-27.5311	24.78659	1-b
2017	477/17	Bat-eared fox	South Africa	North West	-26.6181	25.65319	1-b
2017	480/17	Black-backed jackal	South Africa	North West	-27.1887	25.32931	1-b
2017	466/17	Black-backed jackal	South Africa	North West	-26.7167	27.1	1-b
2017	483/17	Black-backed jackal	South Africa	North West	-26.3138	26.89865	1-b
2017	502/17	Black-backed jackal	South Africa	North West	-26.7167	27.1	1-b
2017	503/17	Black-backed jackal	South Africa	North West	-26.7167	27.1	1-b
2017	LPbov354/17	Bovine	South Africa	Limpopo	-24.5917	27.41155	1-b
2018	NWdog44/18	Canine	South Africa	North West	-26.152	26.15968	1-b
2018	NWbbj110/18	Black-backed jackal	South Africa	North West	-27.1718	26.12699	1-b
2018	NWdog121/18	Canine	South Africa	North West	-26.4677	26.83939	1-b
2018	NWbbj135/18	Black-backed jackal	South Africa	North West	-26.9566	24.7284	1-b
2018	NWbbj195/18	Black-backed jackal	South Africa	North West	-26.2833	26.8	1-b
2018	NWdog270/18	Canine	South Africa	North West	-25.354	26.53009	1-b
2018	NWdog293/18	Canine	South Africa	North West	-25.1334	26.86546	1-b
2018	NWbov299/18	Bovine	South Africa	North West	-27.4377	25.13069	1-b
2018	NWbbj343/18	Black-backed jackal	South Africa	North West	-27.914	25.16111	1-b
2018	NWbov382/18	Bovine	South Africa	North West	-26.152	26.15968	3

2018	NWbbj387/18	Black-backed jackal	South Africa	North West	-26.8097	27.28492	1-b
2018	NWdog391/18	Canine	South Africa	North West	-26.8351	27.04304	1-b
2018	NWdog405/18	Canine	South Africa	North West	-25.8963	27.42684	1-b
2018	NWdog420/18	Canine	South Africa	North West	-25.605	27.91	1-b
2018	NWovi429/18	Ovine	South Africa	North West	-26.4748	27.06278	1-b
2018	NWjac455/18	Jackal	South Africa	North West	-26.152	26.15968	1-b
2018	NWgen516/18	Genet	South Africa	North West	-25.537	26.07512	3
2018	NWbef103/18	Bat-eared fox	South Africa	North West	-26.1944	24.92368	1-b
2019	NWbov76/19	Bovine	South Africa	North West	25.16111	24.17612	1-b
2019	NWbbj96/19	Black-backed jackal	South Africa	North West	-26.3138	26.89865	1-b
2019	NWcap103/19	Caprine	South Africa	North West	-26.8091	26.00538	1-b
2019	NWbov109/19	Bovine	South Africa	North West	-26.9566	24.7284	1-b
2019	NWbov151/19	Bovine	South Africa	North West	-25.1334	26.86546	1-b
2019	NWdog169/19	Canine	South Africa	North West	-26.8091	26.00538	1-b
2019	NWaard171/19	Aardwolf	South Africa	North West	-25.6676	27.24208	1-b
2019	NWdog191/19	Canine	South Africa	North West	-25.6676	27.24208	1-b
2019	NWbbj219/19	Black-backed jackal	South Africa	North West	-25.6676	27.24208	1-b
2019	NWbbj248/19	Black-backed jackal	South Africa	North West	-26.8521	26.66672	1-b
2019	NWjac325/19	Jackal	South Africa	North West	-25.6676	27.24208	1-b
2019	NWbov331/19	Bovine	South Africa	North West	-25.354	26.53009	1-b
2019	NWbov379/19	Bovine	South Africa	North West	-26.9333	25.41667	1-b
2019	NWbov380/19	Bovine	South Africa	North West	-27.2231	25.27706	1-b
2019	NWbov428/19	Bovine	South Africa	North West	-26.2	25.9	3
2019	GPbov309/19	Bovine	South Africa	Gauteng	-26.0858	27.77515	1-b

3.3.3 Phylogenetic analysis of the cytoplasmic domain of the glycoprotein and G-L intergenic region for RABV sequences from the North West province of South Africa

Phylogenetically, the RABV sequences included in the molecular epidemiological investigation of the G-L intergenic region created branching clusters similar to those observed for the partial N gene sequences (**Section 3.3.2**) by forming four distinct clades (A – D) with each clade supported by high posterior probabilities (**Figure 3.5**).

Clade A consisted of RABV sequences (*n = 30*) collected solely from within the NW province of South Africa and consisted of the sequences collected from the following species: canine (*n = 3*), bovine (*n = 15*), black-backed jackal (*n = 7*), bat-eared fox (*n = 2*), caprine (*n = 1*), ovine (*n = 1*), and an unspecified jackal species (*n = 1*). Similar to what was observed with the partial N-gene, the RABV sequences forming part of Clade A were predominantly geographically limited to the western parts of the NW province (**Figure 3.5; Figure 3.6**).

Clade B could be divided into five sub-clades (Sub-clades B-I – B-V), each representing a geographically defined and independent endemic cycle. Sub-clade B-I consisted of RABV sequences originating from black-backed jackals (*n = 9*), canine (*n = 2*), ovine (*n = 1*), and an unspecified jackal species (*n = 1*) (**Figure 3.5**). These sequences were all from the Dr Kenneth Kuanda district in the NW, with only one sequence originating from the adjacent Ngaka Modiri Molema district of the province (**Figure 3.7**). Sub-clade B-II consisted of RABV sequences collected from canine (*n = 2*) and caprine (*n = 1*) samples that were from the parts of the NW bordering GP, and extended into the south-western parts of the Ngaka Modiri Molema district of the NW (**Figure 3.7**). The next sub-clade, Sub-clade B-III, consisted of RABV sequences collected from bovine (*n = 1*), aardwolf (*n = 1*), black-backed jackal (*n = 1*), canine (*n = 2*), and an unspecified jackal species (*n = 1*). The geographic locations for all RABV sequences within this sub-clade were restricted to the Bojanala district of the NW (**Figure 3.7**). Sub-clade B-IV consisted of RABV sequences originating from canine (*n = 3*), and a bovine (*n = 1*). All the RABV sequences in this sub-clade spanned between the NW and GP provinces (**Figure 3.7**). The last sub-clade, Sub-clade B-V, consisted of RABV sequences originating from bovine (*n = 4*), canine (*n = 8*) and caprine (*n = 1*) species and were geographically widespread. In fact, the RABV sequences within this sub-clade had originated from the LP, NW and KZN provinces (**Figure 3.7**).

Similar to what was observed for the partial N gene sequences, Clade C consisted of RABV sequences derived from African civets (*n* = 2) and a genet (*n* = 1) originating from Zimbabwe and the northern parts of the NW Province of South Africa (**Figure 3.5**). Unfortunately, RABV sequences from Botswana could not be included in this analysis as there were no G-L intergenic region sequences from the country.

Clade D contained RABV sequences from yellow mongoose (*n = 2*) and bovine (*n = 2*) samples distributed throughout the FS and NW provinces (**Figure 3.5**). The sequences originating from rabies-positive animals from Clade D were situated in the northern parts of the FS, extending into the southern and central parts of the NW (**Figure 3.6**).

Figure 3.5: Maximum clade credibility phylogenetic tree for the cytoplasmic domain and G-L intergenic region sequences sourced from samples in South Africa and Zimbabwe (**Table 3.3**, **Table A3**). The horizontal branch lengths are proportional to the homology between sequences within and between groups and all branches with a posterior probability of 0.75 or less were collapsed. A canine sequence from Namibia (92030NAM) was used to root the tree. The new sequences generated in this study are shown in bold (**Table 3.3**, **Table A3**). All sequences in Clade A and Clade B belong to the Africa 1-b lineage, while the sequences in Clade C and Clade D belong to the Africa 3 lineage (**Table 3.3**).

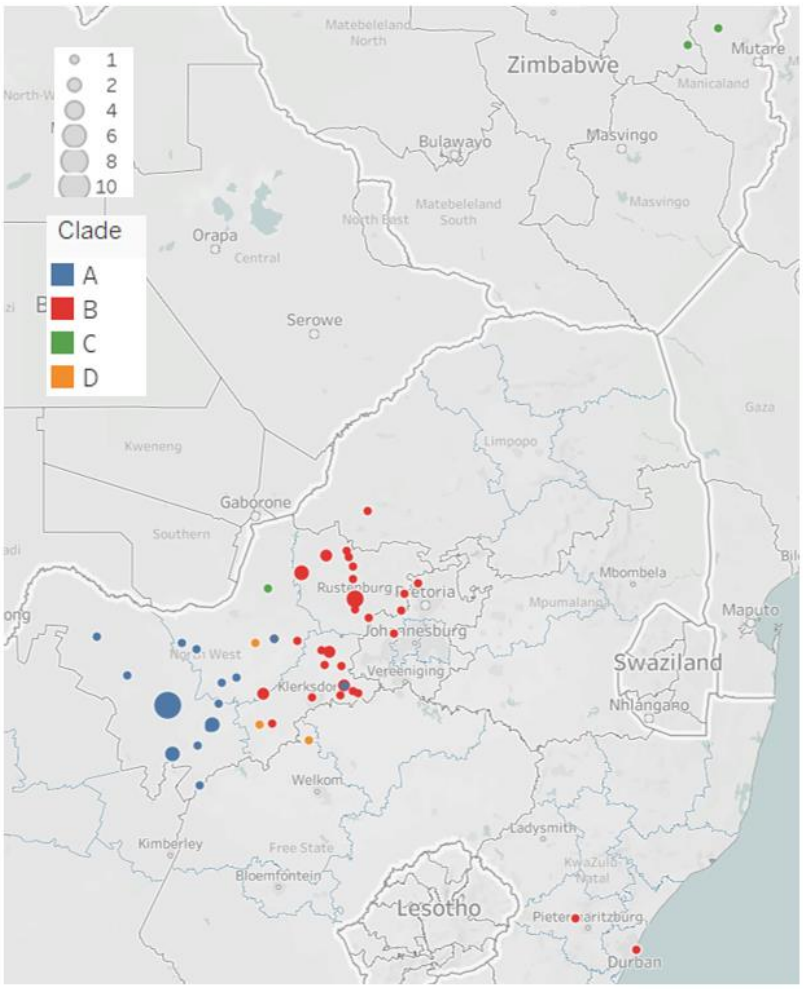

Figure 3.6: Geographic distribution for sequences from each clade from the phylogenetic analysis for the cytoplasmic domain of the glycoprotein gene and adjacent G-L intergenic region for samples included in this study.

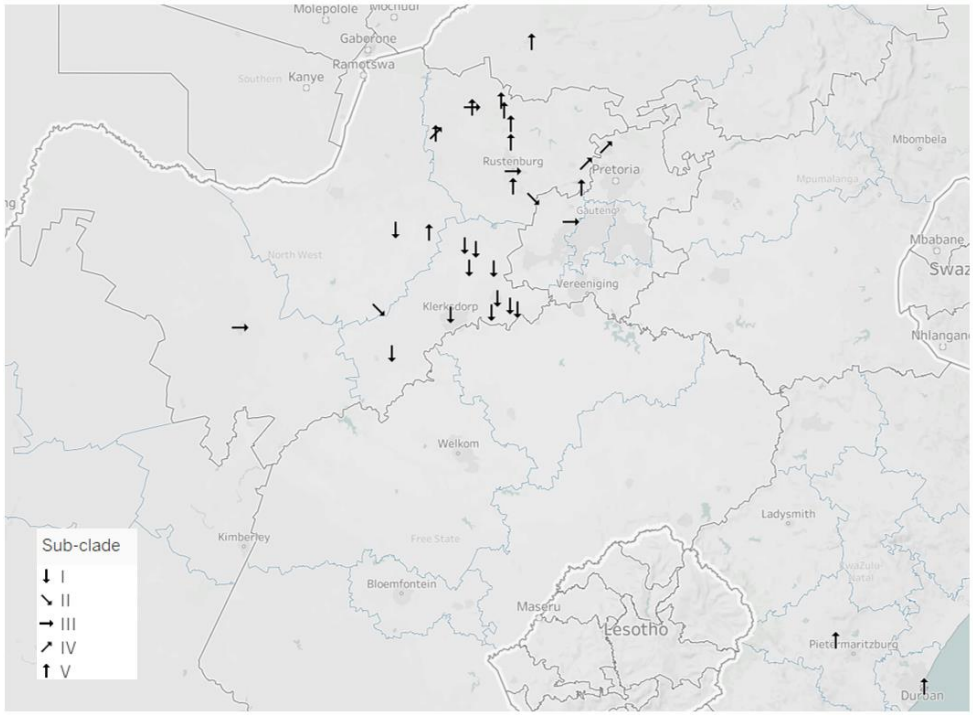

Figure 3.7: Geographic distribution for each of the sequences in the respective sub-clades of Clade B.

3.4 Discussion

The work presented here exemplified the use of molecular epidemiological analyses to update our understanding of epidemiological rabies endemic cycles in specific geographical areas of northern South Africa. This is to the best of our knowledge the first time that such an investigation focussed specifically on the NW Province of South Africa from where epidemiological information on rabies was previously quite limited. The results provided in this investigation indicated that the RABV sequences circulating in the NW province formed part of either the Africa 1b sub-lineage that is predominantly found in East Africa (Hayman *et al.*, 2011; Brunker *et al.*, 2015) or the Africa 3 lineage which is largely limited to the *Herpestidae* family in southern Africa (**Figure 3.3** and **Figure 3.5**).

At a more localized level, the RABV sequences included in this study could phylogenetically be divided into four clades (Clade A–D), while the same branching cluster could be observed for both the G-L intergenic region and the partial N gene region of the RABVs included in the investigation. Clade A consisted almost entirely of RABV sequences collected from sylvatic and livestock species (with 33.3% (*n = 10*) of the sequences in the clade originating from sylvatic species and 56.7% (*n = 17*) originating from livestock) and these were found to be genetically distinct from RABV sequences collected from elsewhere in South Africa or any of the neighbouring countries. This finding suggested that an endemic cycle of sylvatic rabies had become established in the western parts of the NW (**Figure 3.4, Figure 3.6**). In support of the observation, the area's intended land use and historical surveillance data was considered. The western part of the NW (where this cycle of rabies was located) covered a district (Dr Ruth Segomotsi Mompati district) of which approximately 42% of the available land was being used for farming at the time of writing (StatsSA, 2020b). This was an important observation as it had been shown that the incidence of sylvatic rabies cases were higher in areas where commercial and subsistence farming took place due to the low density of domestic dogs that enabled jackal population densities to become high enough to maintain sustained transmission (Van Niekerk, 2010; Badenhorst, 2014) . Furthermore, the historical surveillance data from the western part of the NW indicated that rabies cases in dogs were relatively limited in this part of the province compared to sylvatic rabies cases that were a common occurrence (**Chapter II – Figure 2.5, 2.10 and 2.11**). These findings provide strong evidence that the RABV circulating within the western part of the NW were being maintained within sylvatic reservoir species – with occasional spill-over events to canines.

The RABV sequences that formed part of Clade B had been collected primarily from rabies-positive dogs from various provinces in South Africa (*viz.* the NW, GP, KZN, and LP provinces). This finding suggested that a large endemic cycle of canine-mediated rabies was present in the eastern parts of the NW and that the endemic cycle was also linked with others

that were geographically distinct. To gain improved resolution and, in so doing, better define the broader endemic cycle, the observed clade was broken down into distinct sub-clades in the phylogenetic analysis of the G-L intergenic region.

More specifically, five sub-clades (Sub-clade B-I, B-II, B-III, B-IV, and B-V) were identified. Two of the sub-clades (Sub-clade B-I and B-III) consisted primarily of sylvatic rabies cases that had originated from within the Dr Kenneth Kuanda and Bojanala districts in the eastern parts of the NW, and were thus considered indicative of endemic cycles of sylvatic rabies. Coincidentally, the surveillance data for the NW during a 21-year period (**Chapter II**), indicated that sylvatic rabies cases were often reported for these two districts of the NW province. The clustering observed within these two sub-clades (B-I and B-III) would suggest that two independent endemic cycles of sylvatic rabies was circulating within the eastern parts of the NW (**Figure 3.5**, **Figure 3.7**). The remaining sub-clades (Sub-clade B-II, B-IV, and B-V) consisted primarily of RABV sequences derived from canine and livestock samples from various regions in South Africa (**Figure 3.7**).

The RABV sequences in Sub-clade B-II had originated from canine and caprine samples circulating within eastern parts of the NW (bordering GP) and extended into the south-western regions of the Ngaka Modiri Molema district (**Figure 3.7**). This observation coincides with the known distribution of caprine farming from the NW – suggesting that rabid dogs were largely responsible for the observed infections in goats in the same areas (StatsSA, 2020a). The RABV sequences from Sub-clade B-IV indicated the presence of an independent canine endemic rabies cycle, circulating within the bovine and canine populations in the western regions of the Bojanala district (**Figure 3.7**). The last sub-clade, Sub-clade B-V, contained RABV sequences that came from the NW, LP, GP, and KZN provinces – indicating that endemic cycles of canine rabies from the NW could be linked to endemic rabies cycles in many provinces of South Africa, including the GP province where an outbreak of canine rabies occurred in 2010 (Sabeta *et al.*, 2013). Prior to 2010, sylvatic rabies cases in the GP province were predominantly found in the outlying rural areas of the province, and these cases could be linked to the movement of infected individuals between the NW and GP provinces (Sabeta *et al.*, 2013). This observation is expected as this inter-provincial movement of animals can be seen throughout SA (Ngoepe *et al*., 2009; Mkhize *et al.*, 2010; Sabeta *et al*., 2011b; LeRoux *et al.*, 2018).

The genetic homology observed between the RABV sequences from Sub-clades B-II, B-IV and B-V indicated that interprovincial movement of humans and their companion animals affected the spread and distribution of rabies throughout the country. While the inter-provincial and cross-border movement of humans and their companion animals was not a novel

observation – with various published studies finding similar results – the findings in this study again highlighted that the long-range movement of infected animals between provinces in South Africa is far more widespread than originally thought (Bingham, 2005; Ngoepe, Sabeta and Nel, 2009; Mkhize *et al.*, 2010; Sabeta *et al.*, 2013).

Clade C indicated that there was genetic homology for the RABV sequences collected from civet species in the western parts of Zimbabwe, jackal species in the southern parts of Botswana and a genet in the norther parts of the NW (**Figure 3.6**). During a previous investigation the molecular epidemiology of rabies in Zimbabwe, the researchers found strong evidence that a sylvatic cycle of rabies was being maintained by the civet populations in Zimbabwe (Sabeta *et al.*, 2020). The results of our investigation would suggest that the sylvatic cycle was not only be limited to the civet populations of Zimbabwe, but also extended across Zimbabwe into South Africa and Botswana. This is, however, a speculative observation as there were no additional RABV sequences from those specific locations in South Africa and Botswana to include in our investigation. As a result, it was not possible to determine whether the jackals in Botswana and genets in South Africa were maintaining the endemic cycle or were incidental hosts that encountered another rabid animal.

Clade D consisted solely of sequences of the mongoose variant of the RABV that had been collected from both mongoose and bovine species originating from the NW and FS provinces (**Figure 3.4** and **Figure 3.6**). Based on these findings, it would appear that the mongoose variant of the RABV extends into the NW where it coincides with the natural home-range of mongoose species in those regions.

The work presented in this chapter not only updated our understanding of the molecular epidemiology of rabies in the NW, but also highlighted the genetic relatedness between samples collected in various provinces within South Africa and its neighbouring countries. In addition, we also provided strong evidence in support of the establishment of at least three endemic cycles of sylvatic rabies across different parts of the NW. One of these sylvatic cycles was restricted to the western parts of NW where historical surveillance data suggested most of the sylvatic rabies cases would be found (**Chapter II**). The remaining two sylvatic rabies cycles could be found in the eastern regions of NW where the distribution of sylvatic and canine rabies was found to overlap. Despite this, the results of our investigation suggested that the sylvatic species within the eastern part of the provinces were able to maintain rabies independently from dogs in the same geographical area – impacting future rabies control activities as a result thereof. Lastly, we speculated that a fourth endemic cycle of sylvatic rabies, which appeared to extend between Zimbabwe, Botswana and South Africa might also have become established in the northern parts of the NW Province.

Chapter IV

Molecular epidemiology of rabies in the Limpopo province of South Africa

4.1 Introduction

The LP Province is situated in the northernmost regions of South Africa and shares national borders with the NW, GP and MP provinces while also sharing political borders with Mozambique, Zimbabwe, and Botswana (**Figure 4.1**). While rabies has been endemic to the province since the 1950s (Swanepoel *et al*., 1993; Sabeta *et al*., 2011), historical rabies surveillance data (**Chapter II**) suggests that canine rabies cases were predominantly observed in the northern and north-eastern districts of the province. These could be associated with the major rural settlements of the province while sylvatic rabies cases were found in the western half of the province, associated with predominantly communal and commercial farming areas (**Chapter II**).

Figure 4.1: Districts of the LP province (Municipalities of South Africa, 2021)

To date, various studies have considered surveillance and molecular epidemiological data from this province, focussed on the spread of canine rabies. In one such study, the researchers not only indicated that that an endemic cycle of dog-mediated rabies had become established in the province but also speculated – using surveillance and molecular epidemiological analyses – that rabies was being maintained independently in the black-backed jackal populations in the province, occasionally causing spill-over into cattle and susceptible domestic dog populations (Zulu *et al*., 2009; Mkhize *et al.*, 2010). In another study, which investigated rabies trends in LP to establish a relationship between canine rabies cases

within the province, it was speculated that rabies in the black-backed jackal populations in the Waterberg district (**Figure 4.1**) was distinct and independent when compared to dog rabies cases in that region (Sabeta *et al.*, 2011). In evidence of the fact, there was a decrease in the number of reported rabies cases in dogs between 2005 and 2007 as a result of dog vaccination campaigns (Cohen *et al*., 2007; Sabeta *et al*., 2011), but the number of rabies cases in the jackal population did not reciprocate this decline – despite ongoing surveillance in the animal populations during the same time period (Sabeta *et al.*, 2011). In addition to the molecular epidemiological investigation of rabies within the province specifically, other studies have indicated the movement of rabies-infected animals between LP and the neighbouring countries of Zimbabwe and Mozambique – which further complicated rabies control and surveillance efforts in the province (Zulu *et al*., 2008; Coetzer *et al.*, 2017).

Although the molecular epidemiology of rabies in LP has been studied before, the aim of this chapter was to not only update our current understanding of the endemicity of rabies within the province, but to also improve our understanding of the molecular epidemiology of sylvatic rabies – in relation to canine-mediated rabies – within the LP Province.

4.2 Materials and Methods

4.2.1 Sample cohort

A cohort of contemporary rabies-positive samples (*n = 56*) from various regions within the LP province were chosen for inclusion in this study based on species involved and geographic location of rabies-positive samples collected through routine surveillance between 2017 and 2019.

The geographic distribution for all sequences included in the phylogenetic analyses were visualised using the Tableau software package (version 2020.4.1, Seattle, USA) and relied on the use of geographic coordinates associated with each sample.

4.2.2 RNA extractions

Total RNA was extracted from rabies-positive brain (*n = 56*) according to the manufacturer's instructions as previously described (**Section 3.2.2**).

4.2.3 Reverse transcription of nucleic acids

The reverse transcription of the isolated total RNA was done using an established protocol (Markotter *et al.*, 2006) described previously (**Section 3.2.3**).

4.2.4 Polymerase chain reaction of nucleic acids

4.2.4.1 Polymerase chain reaction amplification of the partial nucleoprotein gene

The partial N gene region was amplified for all samples (*n = 56*) using the modified protocol and standard primer set as previously described (**Section 3.2.6**).

4.2.4.2 Polymerase chain reaction amplification of the G-L intergenic region

As with the partial N gene region, the G-L intergenic gene region was amplified for all samples included in this study (*n = 56*) using a defined protocol and primer set as previously discussed (**Section 3.2.4**).

4.2.5 Agarose gel electrophoresis and PCR clean-up

A 45 µl aliquot of each of the PCR-positive products for both the G-L intergenic region and partial N gene were analysed by agarose gel electrophoresis under UV light and the resulting bands were excised from the agarose gel using a sterile scalpel blade before being transferred to a sterile 1.5 ml microcentrifuge tube (Merck) (**Section 3.2.7**). The excised PCR bands were purified using an established protocol described in **Section 3.2.8**.

4.2.6 Sanger Sequencing

The forward (5'-3') and reverse (3'-5') strands of all the amplified nucleic acid was subjected to Sanger sequencing and precipitated reaction at Inqaba Biotec™ (Pretoria, South Africa) using the ABI Prism 3500XL Genetic Analyzer (ThermoFisher). The final consensus sequences (*n = 56*) were trimmed to 405 nt for the partial N gene and 592 nt for the G-L intergenic region using the CLC Main Workbench software (version 20.0.1). The trimmed consensus sequences, representing the G-L intergenic region and partial N gene of the RABV genome for samples from the LP province (*n = 56*), were submitted to the NCBI GenBank and allocated unique accession numbers (G-L: MW343803 – MW343858; N: MW343913 – MW343968; **Table A4, A5**).

4.2.7 Phylogenetic analysis

The phylogenetic analysis included the 56 RABV sequences sourced from the LP province as described in this study as well as previously published G-L intergenic region sequences (*n = 14*) and partial N gene sequences (*n = 12*) that were reported from South Africa (LP and NW provinces), the neighbouring countries of Zimbabwe, Mozambique, Botswana, and the East African country of Tanzania (**Table A4, A5**). As was the case in the previous chapter (**Chapter III**), it was not possible to undertake the phylogenetic analyses with the exact same panel of RABVs for both the G-L intergenic and partial N gene regions. In this investigation, there were no comparable published G-L intergenic region sequences available for samples sourced from the NC Province in South Africa, Botswana or Zimbabwe. As such, it was not possible to include comparative sequences (collected from samples in the same geographic area and time period) for those N-gene sequences and they were thus omitted from the phylogenetic analysis of the G-L intergenic region.

As such, two separate alignments were created in this investigation – one for the partial N gene (*n = 85*) and one for the G-L intergenic region (*n = 78*) sequences. The sequences for both datasets were aligned using the ClustalW subroutine of the MEGA X software package, and the best-fitting DNA substitution models (partial N gene: TIM1+I; G-L intergenic region: TIM1+G) were determined using the JModel software package (version 2.1.10) using the Akaike's information criterion (AIC).

The final phylogenetic analysis for both gene regions was undertaken using a Bayesian Markov Chain Monte Carlo (MCMC) method in the BEAST software package (version 2.6.0) (Drummond *et al*., 2012). The phylogenetic analysis relied on three independent Markov chains sampled for 10 million states and a sampling frequency of 10,000 was combined after discarding at least a 10 per cent burn. The posterior distributions were subsequently inspected using the Tracer software (version 1.7.1) to ensure adequate mixing and convergence before the associated statistics were summarised as a maximum clade credibility tree and visualised using the FigTree software (version 1.4.4).

4.3 Results

4.3.1. Sample cohort

A cohort of 56 rabies-positive brain samples were selected for inclusion in the molecular epidemiological investigation of rabies in the LP province (**Figure 4.2; Table A4, A5**). The animal species selected for the investigation had been collected during routine surveillance and included the following: black-backed jackal (*n = 1*), bovine (*n = 8*), canine (*n = 43*), unspecified jackal species (*n = 3*) and ovine (*n =1*) species (**Table A4, A5**; **Appendix**

materials). The inclusion of more sylvatic samples would have been preferred for the purpose of this study; however, no other contemporary rabies-positive sylvatic samples were available for study between 2017 and 2019.

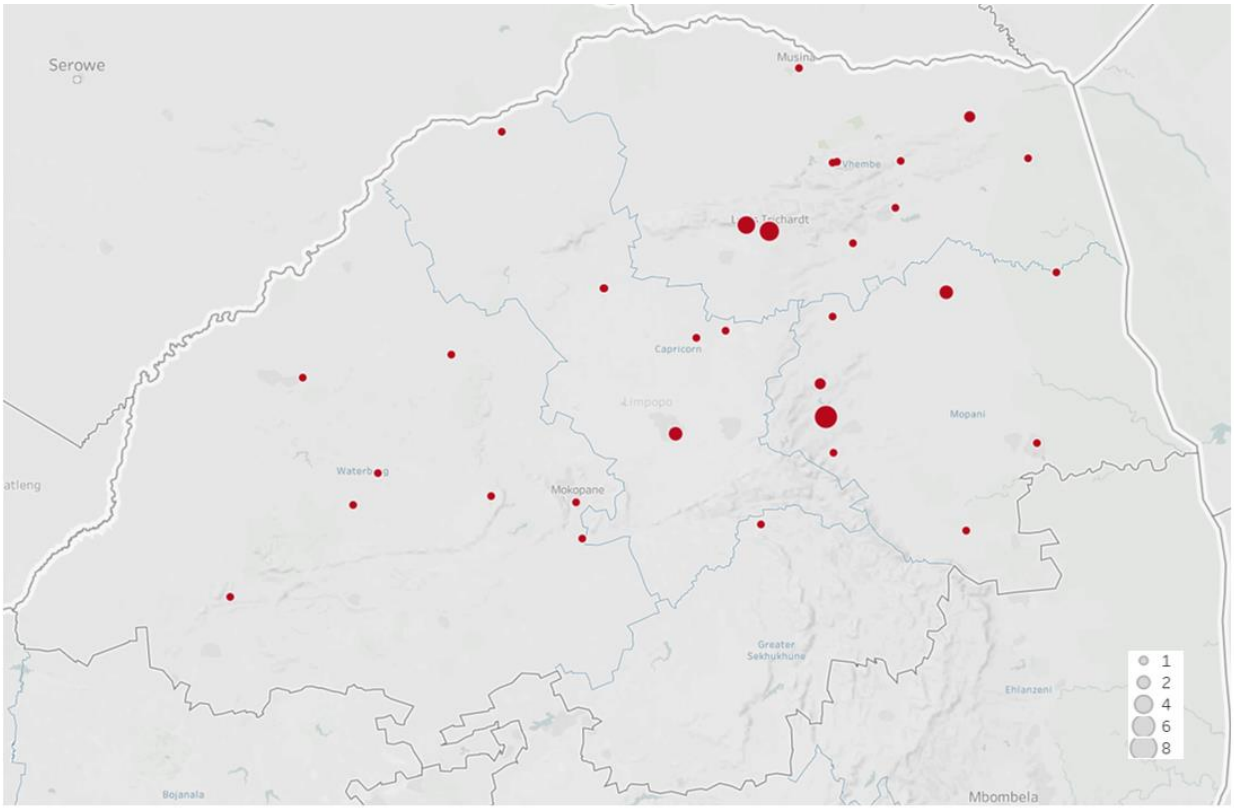

Figure 4.2: Geographic locations of the contemporary rabies-positive samples included in this study from the LP province (*n = 56*)

4.3.2 Phylogenetic analysis of the partial nucleoprotein gene for RABV sequences derived from the Limpopo province

Phylogenetically, the sequences included in the analysis of the partial nucleoprotein gene could be divided into three distinct clades (Clades A-C) (**Figure 4.3** and **Figure 4.4**). Clade A consisted of RABV sequences from the LP Province of South Africa and Tanzania in East Africa (**Figure 4.3).** Clade B consisted of RABV sequences collected from the NW and LP provinces of South Africa, Mozambique, and Zimbabwe. The last clade, Clade C, consisted of RABV sequences that had been obtained from Botswana, and the LP and NC provinces of South Africa (**Figure 4.3**).

Clade A consisted of RABV sequences collected from the LP Province of South Africa (*n = 1*) and Tanzania (*n = 3*). Interestingly, the clustering observed in this clade indicated genetic homology between a contemporary RABV sequence (LPdog307/19) that originated from a

rabies-positive dog in LP and RABV sequences collected from a wild cat (*Felis lybica, n= 1*) and a dog (*n = 1*) in the Serengeti region of Tanzania (**Figure 4.3, Figure 4.4**).

The majority of RABV sequences included in this investigation formed part of Clade B, which contained RABV sequences sourced from canine (*n = 46*), bovine (*n = 5*), black-backed jackal (*n = 6*), aardwolf (*n = 1*), caprine (*n = 1*), civet (*n = 2*) and an unspecified jackal species (*n = 1*) (**Figure 4.3**). The RABV sequences from LP in this clade (Clade B) were predominantly geographically limited to the eastern parts of the province, with this investigation suggesting that the RABV sequences that had originated from the eastern parts of the LP province shared genetic relatedness with those collected from the NW province of South Africa as well as the neighbouring country of Zimbabwe (**Figure 4.4**).

Clade C consisted of RABV sequences collected from canine, sylvatic and livestock species in the LP province (**Figure 4.3**). The RABV sequences in this clade originated from samples collected from black-backed jackals (*n = 3*), equine (*n = 1*), bovine (*n = 5*), ovine (*n = 1*), bat-eared fox (*n = 2*), canine (*n = 3*), and unspecified jackal species (*n = 4*) (**Figure 4.3**). The RABV sequences from the LP province in Clade C were geographically limited to the western parts of the province, with this investigation suggesting that these RABV sequences that had originated from the western parts of LP shared genetic relatedness with RABV sequences collected from the NC Province of South Africa as well as the neighbouring country of Botswana (**Figure 4.4**).

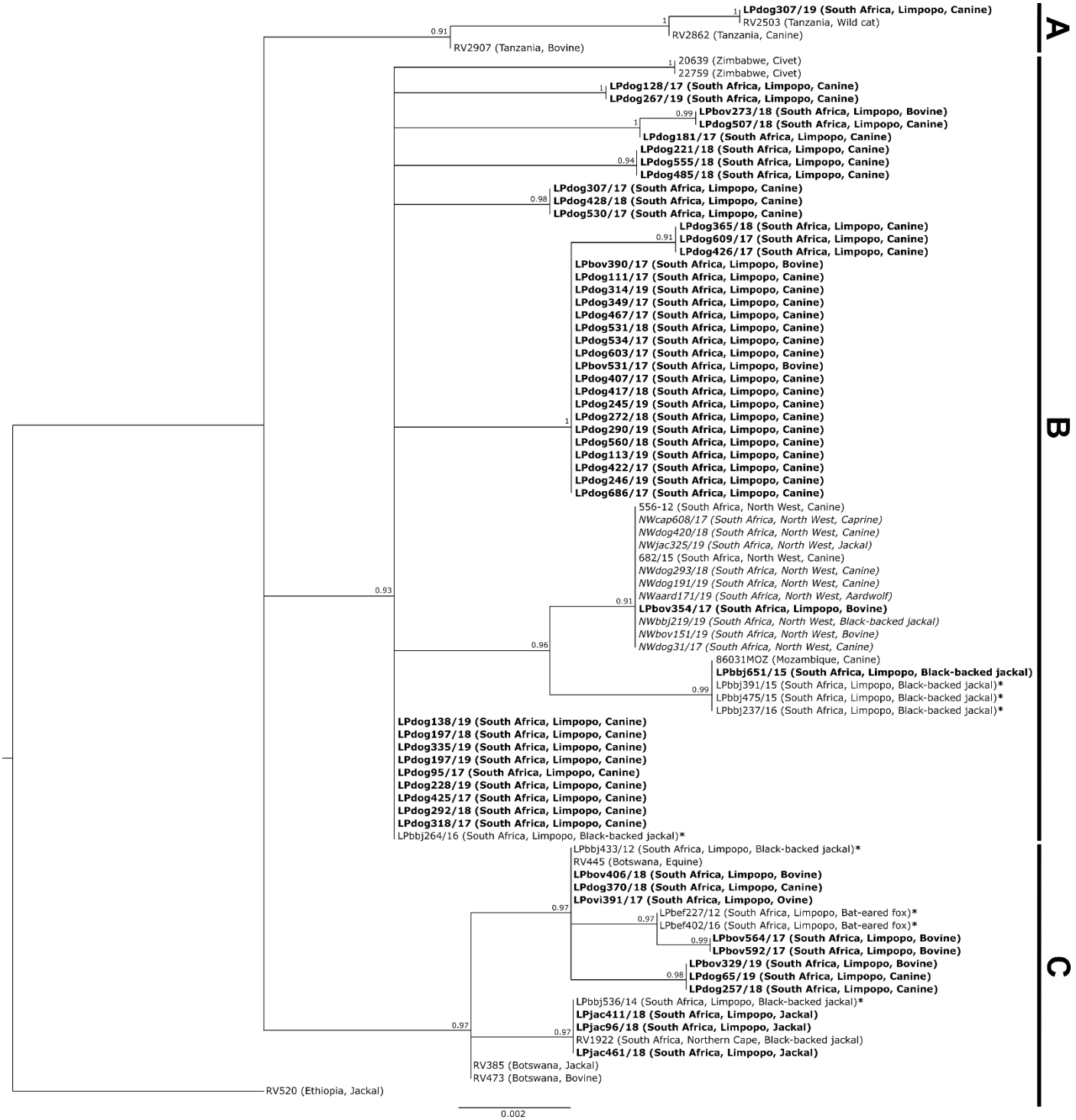

Figure 4.3: Maximum clade credibility phylogenetic tree for the partial N gene region sequences sourced from samples in South Africa, Botswana, Mozambique, Tanzania, and Zimbabwe (**Table 4.1**, **Table A4**). The newly generated sequences in this study are shown in bold. (* denotes sequences that were sequenced in this study for which only previously published G-L intergenic region sequences were available; sequences in italics were generated in Chapter III). All sequences belong to the Africa 1-b lineage (**Table 4.1**).

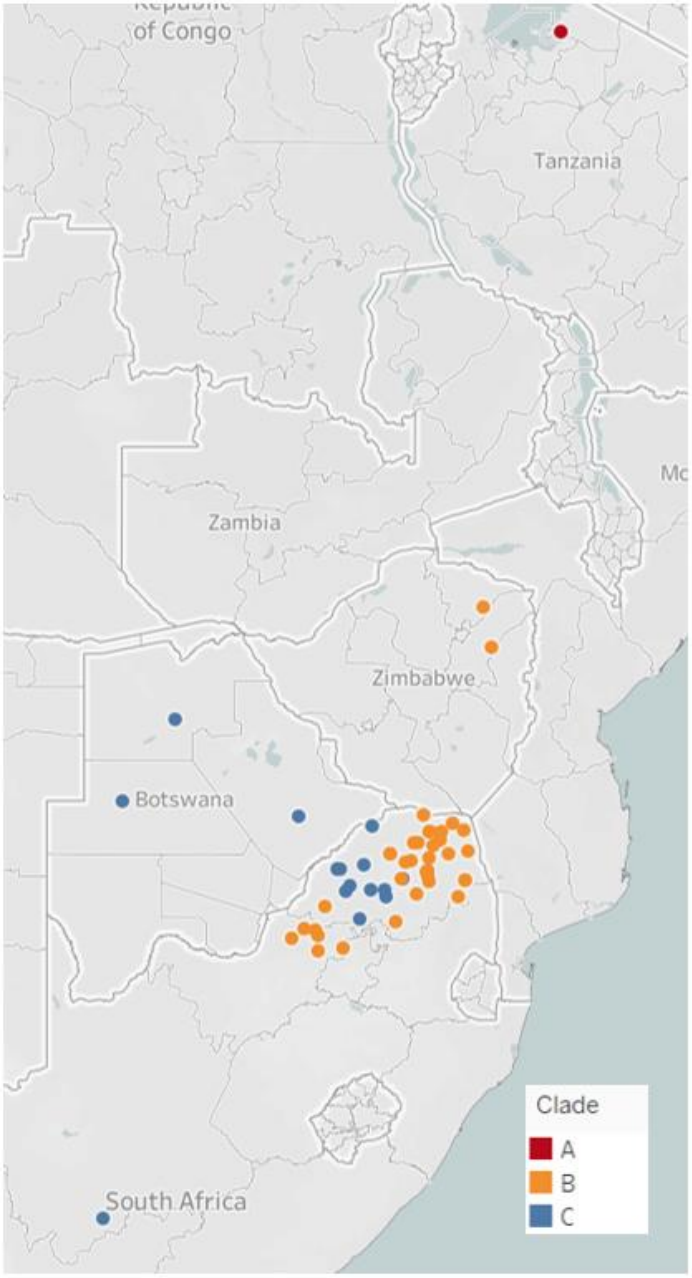

Figure 4.4: Geographic distribution for sequences from each clade from the phylogenetic analysis for the partial N gene sequences for samples included in this study. The geographic locations for the sample LPdog307/19 (Clade A) and 86031MOZ (Clade B) were not defined.

Table 4.1: Sample cohort for all RABV sequences included in the phylogenetic analyses for samples from rabies-positive animals originating from LP included in this study

Year Sampled	Sample Number	Species	Country	Province	Latitude	Longitude	Africa Lineage
1986	86031MOZ	Canine	Mozambique	Unknown	Unknown	Unknown	1-b
1988	385	Jackal	Botswana	Ghanzi	-22	22	1-b
1991	445	Horse	Botswana	Maun	-19.9833	23.4167	1-b
1991	473	Bovine	Botswana	Serowe	-22.3875	26.7108	1-b
1992	20639	African civet	Zimbabwe	Macheke	-18.139	31.8493	1-b

1994	22759	African civet	Zimbabwe	Shamva	-17.1237	31.6415	1-b
1997	RV1922	Black-backed jackal	South Africa	Northern Cape	-31.9157	21.5134	1-b
2012	556/12	Canine	South Africa	Mogwase	-25.2775	27.2161	1-b
2012	433/12	Black-backed jackal	South Africa	Limpopo	-23.9045	29.4689	1-b
2012	LPbef227/12	Bat-eared fox	South Africa	Limpopo	-23.667	27.8077	1-b
2014	LPbbj536/14	Black-backed jackal	South Africa	Limpopo	-24.884	28.3287	1-b
2015	LPbbj391/15	Black-backed jackal	South Africa	Limpopo	-24.351	30.9577	1-b
2015	LPbbj475/15	Black-backed jackal	South Africa	Limpopo	-23.92	29.4554	1-b
2015	LPbbj651/15	Black-backed jackal	South Africa	Limpopo	-24.9663	29.2907	1-b
2015	682/15	Canine	South Africa	North West	-25.354	26.5301	1-b
2016	LPbbj237/16	Black-backed jackal	South Africa	Limpopo	-23.943	31.1411	1-b
2016	LPbbj264/16	Black-backed jackal	South Africa	Limpopo	-23.743	30.1168	1-b
2016	LPbef402/16	Bat-eared fox	South Africa	Limpopo	-23.666	27.7448	1-b
2017	LPdog95/17	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2017	LPdog111/17	Canine	South Africa	Limpopo	-23.6934	30.14	1-b
2017	LPdog128/17	Canine	South Africa	Limpopo	-22.9456	30.485	1-b
2017	LPdog181/17	Canine	South Africa	Limpopo	-23.0439	29.9032	1-b
2017	LPdog307/17	Canine	South Africa	Limpopo	-23.4667	29.7	1-b
2017	LPdog318/17	Canine	South Africa	Limpopo	-23.0949	30.2908	1-b
2017	LPdog349/17	Canine	South Africa	Limpopo	-23.943	31.1411	1-b
2017	LPbov354/17	Bovine	South Africa	Limpopo	-24.5917	27.4116	1-b
2017	LPbov390/17	Bovine	South Africa	Limpopo	-23.2201	31.2288	1-b
2017	LPovi391/17	Ovine	South Africa	Limpopo	-22.6215	28.6665	1-b
2017	LPdog407/17	Canine	South Africa	Limpopo	-23.4093	30.1954	1-b
2017	LPdog422/17	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2017	LPdog425/17	Canine	South Africa	Limpopo	-23.0176	29.7984	1-b
2017	LPdog426/17	Canine	South Africa	Limpopo	-22.7547	30.1936	1-b
2017	LPdog467/17	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2017	LPdog530/17	Canine	South Africa	Limpopo	-23.2861	29.1396	1-b
2017	LPbov531/17	Bovine	South Africa	Limpopo	-23.3025	30.7187	1-b
2017	LPdog534/17	Canine	South Africa	Limpopo	-24.2848	29.8638	1-b
2017	LPbov564/17	Bovine	South Africa	Limpopo	-24.1667	28.6167	1-b
2017	LPbov592/17	Bovine	South Africa	Limpopo	-24.0685	28.0939	1-b
2017	LPdog603/17	Canine	South Africa	Limpopo	-23.3025	30.7187	1-b
2017	LPdog609/17	Canine	South Africa	Limpopo	-22.7333	31.1	1-b
2017	LPdog686/17	Canine	South Africa	Limpopo	-22.3488	30.0407	1-b
2017	NWdog31/17	Canine	South Africa	North West	-25.1609	27.163	1-b

2017	NWcap608/17	Caprine	South Africa	North West	-25.1334	26.8655	1-b
2018	LPjac96/18	Jackal	South Africa	Limpopo	-24.2051	27.9787	1-b
2018	LPdog197/18	Canine	South Africa	Limpopo	-23.0176	29.7984	1-b
2018	LPdog221/18	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2018	LPdog257/18	Canine	South Africa	Limpopo	-23.9045	29.4689	1-b
2018	LPdog272/18	Canine	South Africa	Limpopo	-23.0176	29.7984	1-b
2018	LPbov273/18	Bovine	South Africa	Limpopo	-23.0176	29.7984	1-b
2018	LPdog292/18	Canine	South Africa	Limpopo	-22.7457	30.5093	1-b
2018	LPdog365/18	Canine	South Africa	Limpopo	-23.3025	30.7187	1-b
2018	LPdog370/18	Canine	South Africa	Limpopo	-23.6661	27.7448	1-b
2018	LPbov406/18	Bovine	South Africa	Limpopo	-23.5703	28.4341	1-b
2018	LPjac411/18	Jackal	South Africa	Limpopo	-23.9045	29.4689	1-b
2018	LPdog417/18	Canine	South Africa	Limpopo	-23.498	29.5672	1-b
2018	LPdog428/18	Canine	South Africa	Limpopo	-23.288	29.1368	1-b
2018	LPjac461/18	Jackal	South Africa	Limpopo	-24.3474	29.0388	1-b
2018	LPdog485/18	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2018	LPdog507/18	Canine	South Africa	Limpopo	-22.75	30.2167	1-b
2018	LPdog531/18	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2018	LPdog555/18	Canine	South Africa	Limpopo	-23.9833	30.2	1-b
2018	LPdog560/18	Canine	South Africa	Limpopo	-23.0176	29.7984	1-b
2018	NWdog293/18	Canine	South Africa	North West	-25.1334	26.8655	1-b
2018	NWdog420/18	Canine	South Africa	North West	-25.605	27.91	1-b
2019	LPdog65/19	Canine	South Africa	Limpopo	-23.9045	29.4689	1-b
2019	LPdog113/19	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2019	LPdog138/19	Canine	South Africa	Limpopo	-23.0439	29.9032	1-b
2019	LPdog197/19	Canine	South Africa	Limpopo	-23.0439	29.9032	1-b
2019	LPdog228/19	Canine	South Africa	Limpopo	-22.5587	30.828	1-b
2019	LPdog245/19	Canine	South Africa	Limpopo	-23.0439	29.9032	1-b
2019	LPdog246/19	Canine	South Africa	Limpopo	-23.0439	29.9032	1-b
2019	LPdog267/19	Canine	South Africa	Limpopo	-22.5587	30.828	1-b
2019	LPdog290/19	Canine	South Africa	Limpopo	-23.6934	30.14	1-b
2019	LPdog307/19	Canine	South Africa	Limpopo			1-b
2019	LPdog314/19	Canine	South Africa	Limpopo	-23.8332	30.1635	1-b
2019	LPbov329/19	Bovine	South Africa	Limpopo	-24.1944	29.0097	1-b
2019	LPdog335/19	Canine	South Africa	Limpopo	-23.0439	29.9032	1-b
2019	NWaard171/19	Aardwolf	South Africa	North West	-25.6676	27.2421	1-b
2019	NWbbj219/19	Black-backed jackal	South Africa	North West	-25.6676	27.2421	1-b
2019	NWbov151/19	Bovine	South Africa	North West	-25.1334	26.8655	1-b
2019	NWdog191/19	Canine	South Africa	North West	-25.6676	27.2421	1-b
2019	NWjac325/19	Jackal	South Africa	North West	-25.6676	27.2421	1-b
2009	RV2503	Wild Cat	Tanzania	Serengeti	-2.03961	33.714	1-b
2011	RV2862	Canine	Tanzania	Serengeti	-2.03961	33.714	1-b
2011	RV2907	Bovine	Tanzania	Serengeti	-2.03961	33.714	1-b

4.3.3 Phylogenetic analysis of the cytoplasmic domain of the glycoprotein and G-L intergenic region for sequences from the Limpopo province

Phylogenetically, the RABV sequences included in the analysis of the G-L intergenic region could be divided into five distinct clades (Clades A-E) (**Figure 4.5**). Although not identical, the clustering of the RABV sequences included in the molecular epidemiological investigation of the G-L intergenic region were similar to what was observed for the partial N gene sequences (**Section 4.3.2**).

Clade A consisted of a RABV sequences collected from the LP Province of South Africa (*n = 1*) and Tanzania (*n = 3*) (**Figure 4.5**). In this clade, a canine (*n = 1*) sample from LP showed genetic homology with RABV sequences collected from a wild cat (*n = 1*), canine (*n = 1*) and bovine (*n = 1*) in the Serengeti district of Tanzania.

Clade B consisted of RABV sequences that had solely been derived from the eastern parts of LP (**Figure 4.6)** and consisted of RABV sequences that originated from canine (*n = 39*), bovine (*n = 3*) and a black-backed jackal (*n = 1*) sample (**Figure 4.5**).

Clade C consisted of RABV sequences that had been derived from samples collected from black-backed jackals (*n = 6*), bovine (*n = 2*), canine (*n = 7*), caprine (*n = 1*), aardwolf (*n = 1*) and an unspecified jackal species (*n = 1*) (**Figure 4.5**). The sequences in this clade had originated from Mozambique, the southern parts of the LP province and the areas of the NW province neighbouring the LP province (**Figure 4.6)**.

Clade D was restricted to the Waterberg and Capricorn district in the western parts of the LP Province and consisted of samples collected from canine (*n = 3*), bovine (*n = 2*), ovine (*n = 1*) and a black-backed jackal (*n = 1*) sample (**Figures 4.5, 4.6**).

The last clade, Clade E, was restricted to the Waterberg district in the west of the province and consisted of RABV sequences that had originated from samples collected from bat-eared fox (*n = 2*), bovine (*n = 2*) and unspecified jackal species (*n = 3*) (**Figures 4.5, 4.6**).

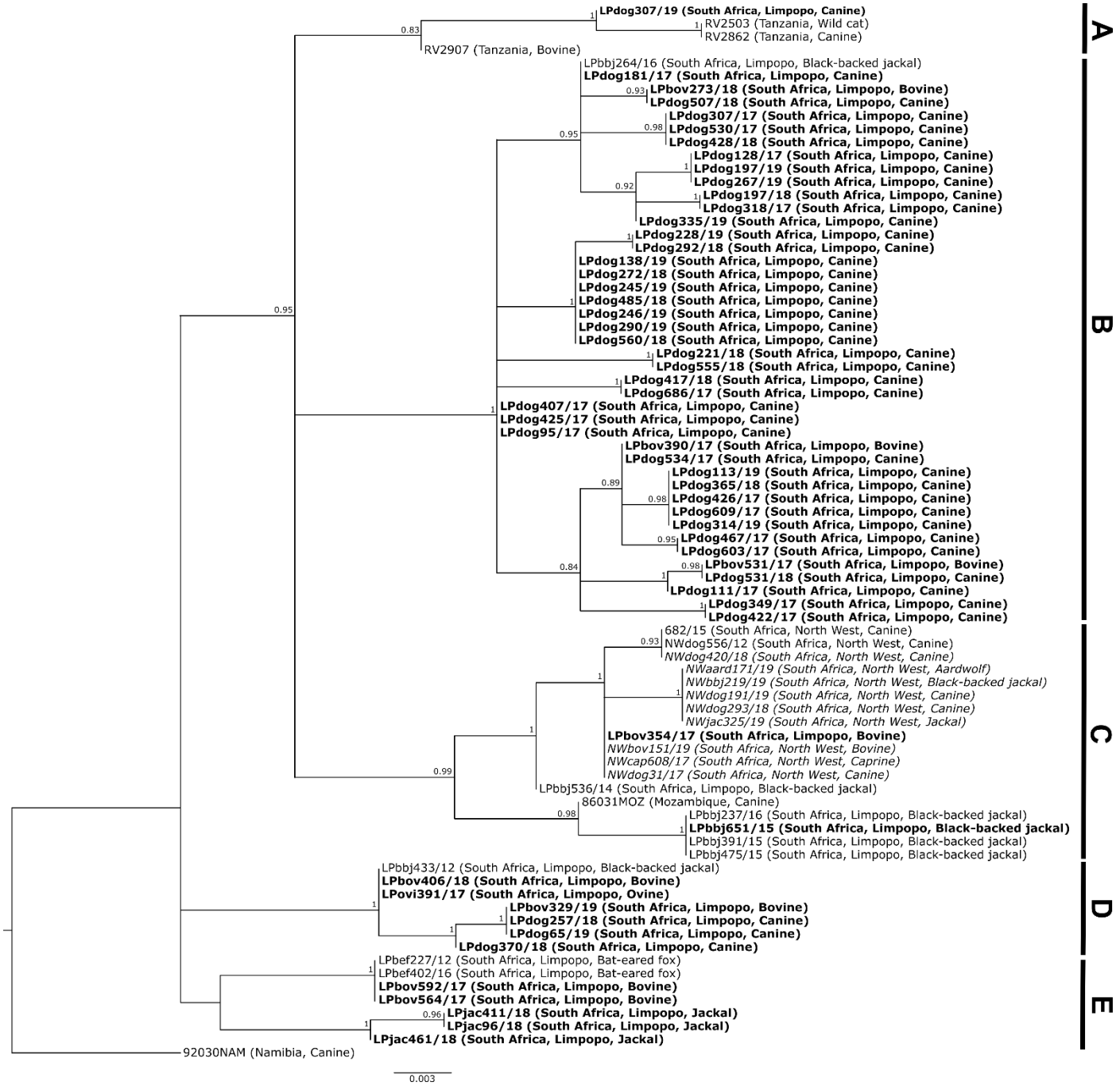

Figure 4.5: Maximum clade credibility tree for the G-L intergenic sequences for RABV sequences originating from various regions in South Africa, Mozambique, Zimbabwe, and Tanzania (**Table 4.1**; **Table A5**). The horizontal branch lengths are proportional to the similarity of the sequences within and between groups; all branches with a posterior probability of 0.75 or less were collapsed. A canine RABV sequence from Namibia (92030NAM) was used to root the tree. The new sequences generated in this study have been indicated in a bold font while sequences in italics were generated in Chapter III. All sequences belong to the Africa 1-b lineage (**Table 4.1**).

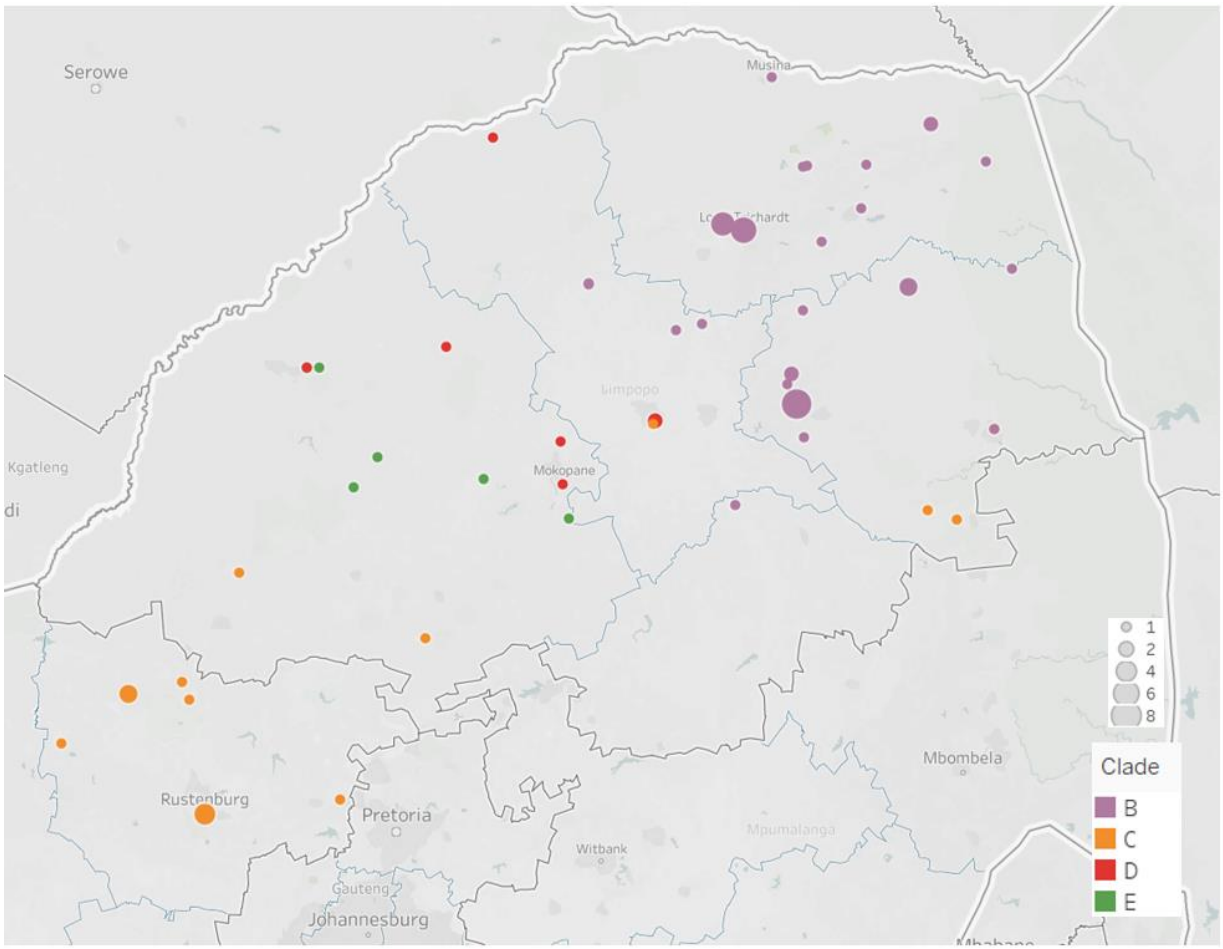

Figure 4.6: Geographic distribution for sequences (excluding those originating from Tanzania) included in the phylogenetic analysis of the G-L intergenic region, according to their clades (**Table A5**).

4.4 Discussion

The molecular epidemiology of rabies in LP has been documented extensively throughout the years with most studies focussing on defining endemic cycles of dog-mediated rabies (Swanepoel *et al.*, 1993; Cohen *et al.*, 2007; Zulu *et al*., 2009; Sabeta *et al.*, 2011b). The molecular epidemiological analyses in this study not only provided further evidence for canine endemic rabies cycles circulating within LP, but also provided insights into the interaction between canine and sylvatic species within the province (**Figure 4.3, Figure 4.5).** Furthermore, the molecular epidemiological information produced in this investigation highlighted a few points of interest.

Briefly, the RABV sequences included in the phylogenetic analysis of the partial N gene analysis formed three distinct clades which consisted of RABV sequences that all belonged to the Africa 1-b lineage found circulating within canine populations in southern and eastern Africa (Clades A – C; **Figure 4.3**). Clade A highlighted the genetic relatedness of RABV sequences from the LP Province of South Africa and Tanzania, while Clade B consisted mainly

of RABV sequences sourced from canines originating from Mozambique, the eastern half of LP and the NW Province of South Africa. The last remaining clade, Clade C, contained RABV sequences from various species and highlighted the genetic relatedness of RABV sequences from Botswana and the western half of LP and the NC Province of South Africa (**Figure 4.3**).

To gain additional clarity in terms of the genetic relatedness of the RABV sequences included in this investigation we undertook a molecular epidemiological analysis of the G-L intergenic region of the RABV sequences. Indeed, this specific gene region resulted in the sequences clustering into five distinct clades as discussed below (**Figure 4.5**).

The first clade, Clade A, provided the most unexpected finding by highlighting the genetic relatedness between RABV sequences originating from the LP Province of South Africa and the Serengeti district of Tanzania in East Africa (a country that is approximately 3,500 kilometres away). Rabies was first documented in the Serengeti district of Tanzania in the 1970s, after which the occurrence of long-range transmission from within the district had rarely been documented (Magembe, 1985; Siongok and Karama, 1985; Brunker *et al.*, 2015). It was therefore highly unexpected to find genetic homology between a RABV sequences from the LP province (LPdog307/19) and the Serengeti district of Tanzania (**Figure 4.3** and **Figure 4.5**). The genetic relatedness between these RABV sequences highlighted the role that human (and their companion animals) movement play in the long-range transmission of rabies as previously published (Wheeler and Waller, 2008; Shite *et al.*, 2015; Colombi *et al.*, 2020).

The second clade, Clade B, provides phylogenetic evidence in support of the presence of an endemic cycle of canine rabies that was circulating within the eastern parts of the LP Province. This was not a novel observation as similar findings were observed in previous molecular epidemiological studies that had observed similar geographical trends (Cohen *et al.*, 2007; Zulu *et al.*, 2009; Sabeta *et al*., 2011a), which were further substantiated through the use of empirical surveillance data (**Chapter II**).

Clade C consisted primarily of RABV sequences from the NW and LP provinces in South Africa as well the neighbouring country Mozambique. The presence of a RABV sequence originating from Mozambique further highlights that the transboundary transmission of rabies between the LP Province of South Africa, Mozambique and Zimbabwe, which had previously been discussed and was thus not a novel observation (Zulu *et al*., 2008; Coetzer *et al.*, 2017; Coetzer *et al.*, 2019). Furthermore, the branch clustering of RABV sequences from both canine and sylvatic species in this clade suggested that spill-over infections occur in areas where the geographic areas between these species overlap. There was, however, no strong evidence that suggested that rabies was being maintained independently by the sylvatic species in eastern LP.

We speculated that the divergence in the number of clades observed between the phylogenetic analyses for the G-L intergenic region and the partial N gene was due to the geographic separation of the RABV sequences in Clade D and Clade E (**Figure 4.5, Figure 4.6**). Clade D, which consisted of mostly RABV sequences collected from dogs, indicated that an endemic cycle of canine rabies has become established in the northern parts of the Waterberg district in the West of the province. Clade E, which consisted of RABV sequences collected mostly from black-backed jackal and bat-eared foxes suggested that an endemic cycle of sylvatic rabies was persisting in the central regions of the Waterberg district in the West of the province (**Figure 4.6**). While the maintenance of sylvatic rabies in the western parts of LP had been speculated, this study provides further evidence to support the notion that sylvatic species were able to maintain rabies independently of canine rabies within LP.

In support of the trends observed using the historical surveillance data for the epidemiology of rabies in LP, and other published studies (Cohen *et al.*, 2007; Zulu *et al.*, 2009; Sabeta *et al*., 2011), our molecular epidemiological investigation suggested that endemic cycles of canine-mediated rabies persisted mostly in the eastern parts of LP while also providing evidence that supported the notion that sylvatic species within the westerns parts of the province were able to independently maintain rabies. The work presented in this chapter not only allowed us to update our understanding of the molecular epidemiology of rabies in LP, but also provided additional evidence in support of the presence of endemic cycles of sylvatic rabies circulating in the western parts of LP. In addition, the findings presented here also provided evidence of long-range transmission of RABV-positive animals between South Africa and Tanzania.

Chapter V

Concluding remarks

Canine-mediated rabies still poses a considerable public health threat in more than 120 countries around the world, including South Africa. While South Africa has made considerable progress towards reaching freedom from canine-mediated human rabies (Weyer, 2015), the deadline for self-declaring of freedom by 2030 is fast-approaching. To achieve this goal, a significant proportion of the at-risk dog populations in the country needs to be vaccinated to interrupt disease transmission and maintain herd immunity. In order to achieve this, the South African government should rely on epidemiological data – generated through the reporting of all suspected rabies cases – which would, in turn, allow them to implement resource-considerate disease intervention campaigns in regions where transmission is known to occur. This approach has proven effective and has even led to the elimination of canine rabies in several countries throughout the world (e.g., western European countries and the USA). However, the elimination of canine rabies in these countries saw the persistence of rabies within various sylvatic reservoir species, which pose the risk of re-introducing rabies into immunologically naïve dog populations and preventing canine-mediated rabies from being completely eliminated. Therefore, defining and understanding sylvatic rabies cycles within any given country is imperative as it would allow the governments to better focus their rabies elimination attempts through the use of parenteral vaccinations for dogs and ORVs for sylvatic populations where appropriate and necessary (to limit the likelihood of reintroduction into the dog population).

To the best of our knowledge, this study provided the first in-depth molecular epidemiological investigation of RABV sequences collected from within the NW Province of South Africa. The high degree of genetic similarity between the viruses suggested regular and unrestricted cross-border movement of rabid animals between the NW and other South African provinces (*viz*. GP, KZN and LP provinces) and neighbouring countries (Botswana, Mozambique, and Zimbabwe). In addition, the phylogenetic evidence – coupled with empirical surveillance data collected over the last 21 years – suggested the presence of at least three independent cycles of sylvatic rabies within the NW. A potential fourth endemic sylvatic cycle, ranging from Zimbabwe to South Africa and Botswana, was identified. However, definitive characterisation of this potential cycle would require a significantly larger sample size.

Although the molecular epidemiology of rabies in LP has been described and documented in the past, this was the first study to focus specifically on the interaction between endemic cycles of canine and sylvatic rabies circulating within LP and its neighbouring provinces and countries. From the phylogenetic analyses conducted during this investigation, along with empirical surveillance data, we were able to identify a separate sylvatic endemic rabies cycle circulating within the Waterberg district of LP. While the maintenance of sylvatic rabies was speculated, this study provided evidence that sylvatic species are indeed able to maintain

rabies endemic cycles independent from cycles in domestic dogs. Furthermore, based on the genetic homology of sequences generated in this study, the cross-border movement of rabies infected individuals between LP and neighbouring countries (*viz.* Mozambique, Zimbabwe, and Botswana) and provinces (viz. NW and NC provinces) was highlighted. Interestingly, this study is the first to show that there was a high level of genetic relatedness between RABV sequences collected in the LP Province and Botswana – suggesting unrestricted cross-border transmission of rabies between South Africa and Botswana. This study also showed potential long-range transmission of RABV between South Africa and the Serengeti district in Tanzania, based on viral sequences deposited in the public domain – which, if proven, would highlight the lack of efficient border control measures between countries.

In support of the surveillance data collected over the 21-year period in study, the findings presented here suggested that rabies was maintained by sylvatic populations, primarily black-backed jackals, in the northern parts of South Africa. In addition, the findings also indicated that spill-over infections between sylvatic and domestic species in both provinces (clades that are mostly sylvatic but have a few dogs in them) had occurred in the past. As such, the findings presented here – and from a previous investigation (Sabeta *et al.*, 2007) – suggested that targeted parenteral rabies vaccination of dog populations could, in theory, not be enough to completely eliminate canine-mediated rabies and would most likely have little/no impact on the number of sylvatic rabies cases in the NW and LP provinces specifically. Despite having a seemingly negligible public health impact, the persistence of sylvatic rabies – coupled with the intermittent spill-over infections to co-habiting dog populations as observed throughout this investigation – could, in theory, result in the re-introduction of rabies into immunologically naïve dog populations. Therefore, to eliminate canine-mediated rabies from South Africa, disease control and elimination efforts would have to focus on both the domestic dog (by means of parenteral vaccination) and the sylvatic populations (by means of ORVs) in the provinces where endemic cycles of sylvatic rabies are known to occur. Indeed, Bingham *et al*., (1999) tested the efficacy of the SAG-2 ORV in jackal populations in Zimbabwe and found the ORV to be effective at achieving an adequate level of seroconversion in target populations (Bingham *et al*., 1999) – highlighting the feasibility of using ORV's in southern African countries. In summary, if the government could rely on this two-pronged approach of using parenteral vaccination in conjunction with ORVs, canine-mediated rabies could be eliminated from both domestic and sylvatic species in the country. This would, in-turn, enable the South African government to self-declare freedom from canine-mediated rabies by means of vaccination.

References

2011 Census | Statistics South Africa (no date). Available at: http://www.statssa.gov.za/?page_id=3839 (Accessed: 28 October 2020).

Abellán García, C. *et al.* (2004) 'Rabies in the iberian peninsula', *Historical Perspective of Rabies in Europe and the Mediterranean Basin. Paris: World Organization for Animal Health*, pp. 147–156.

Amarasinghe, G. K. *et al.* (2017) 'Taxonomy of the order Mononegavirales: update 2017', *Archives of Virology*. Springer Vienna, 162(8), pp. 2493–2504. doi: 10.1007/s00705-017-3311-7.

Athingo, R. *et al.* (2020) 'Fighting Dog-Mediated Rabies in Namibia—Implementation of a Rabies Elimination Program in the Northern Communal Areas', *Tropical Medicine and Infectious Disease*. MDPI AG, 5(1), p. 12. doi: 10.3390/tropicalmed5010012.

Aubert, M. F. A. *et al.* (2004) 'Rabies in France, the Netherlands, Belgium, Luxembourg and Switzerland', *Historical perspective of rabies in Europe and the Mediterranean Basin*. Office International des Epizooties Paris, 129, p. 145.

Badenhorst, C. G. (2014) 'The economic cost of large stock predation in the North West Province of South Africa'. University of the Free State.

Baer, G. M. (2007) '1 - The History of Rabies', in Jackson, A. C. and Wunner, W. H. B. T.-R. (Second E. (eds). Oxford: Academic Press, pp. 1–22. doi: https://doi.org/10.1016/B978-012369366-2/50003-8.

Balako, G. *et al.* (2018) 'Rabies Outbreak among Livestock in a Pastoralist Community, Southern Ethiopia', *Ethiopian journal of health sciences*. NLM (Medline), 28(6), pp. 805–808. doi: 10.4314/ejhs.v28i6.16.

Barnard, B. J. H. and Hassel, R. H. (1981) 'Rabies in kudus (Tragelaphus strepsiceros) in South West Africa/Namibia', *Journal of the South African Veterinary Association*. Sabinet, 52(4), pp. 309–314.

Bellan, S. E. *et al.* (2012) 'Black-backed jackal exposure to rabies virus, canine distemper virus, and Bacillus anthracis in Etosha National Park, Namibia', *Journal of Wildlife Diseases*. Wildlife Disease Association, Inc., 48(2), pp. 371–381. doi: 10.7589/0090-3558-48.2.371.

Berndtsson, L. T. *et al.* (2011) 'Factors associated with the success of rabies vaccination of dogs in Sweden', *Acta Veterinaria Scandinavica*. BioMed Central, 53(1), pp. 1–7. doi: 10.1186/1751-0147-53-22.

Bingham, A. *et al.* (1999) *The epidemiology of rabies in Zimbabwe. 2. Rabies in jackals (Canis*

adustus and Canis mesomelas), *Onderstepoort Journal of Veterinary Research*. Available at: https://pdfs.semanticscholar.org/f64d/dd704db6be1e1b4729891faf897e74993bb8.pdf (Accessed: 25 March 2019).

Bingham, J. *et al.* (1999) 'Efficacy of SAG-2 oral rabies vaccine in two species of jackal (Canis adustus and Canis mesomelas)', *Vaccine*. Elsevier, 17(6), pp. 551–558.

Bingham, J. (2005) 'Canine rabies ecology in Southern Africa', *Emerging Infectious Diseases*. Centers for Disease Control and Prevention (CDC), pp. 1337–1342. doi: 10.3201/eid1109.050172.

Bishop, G. C. *et al.* (2010) 'Guide for the Medical, Veterinary and allied Professions'. Department of Agriculture, Forestry, and Fisheries, Republic of South Africa.

Blancou, J. (2004) 'Rabies in Europe and the Mediterranean basin: from antiquity to the 19th century', *Historical perspective of rabies in Europe and the Mediterranean basin. Paris: Organisation for Animal Health*, pp. 15–23.

Bourhy, H. *et al.* (1992) 'Antigenic and molecular characterization of bat rabies virus in Europe.', *Journal of Clinical Microbiology*. American Society for Microbiology Journals, 30(9), pp. 2419–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1401009 (Accessed: 6 May 2019).

Bourhy, H., Kissi, B. and Tordo, N. (1993) 'Taxonomy and evolutionary studies on lyssaviruses with special reference to Africa.', *The Onderstepoort Journal of Veterinary Research*, 60(4), pp. 277–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7777311 (Accessed: 6 May 2019).

Brochier, B. M. *et al.* (1988) 'Use of recombinant vaccinia-rabies virus for oral vaccination of fox cubs (Vulpes vulpes, L) against rabies', *Veterinary Microbiology*. Elsevier, 18(2), pp. 103–108.

Brookes, V. J. *et al.* (2019) 'Challenges to human rabies elimination highlighted following a rabies outbreak in bovines and a human in Punjab, India', *Zoonoses and Public Health*. Wiley-VCH Verlag, 66(3), pp. 325–336. doi: 10.1111/zph.12568.

Brunker, K. *et al.* (2015) 'Elucidating the phylodynamics of endemic rabies virus in eastern Africa using whole-genome sequencing', *Virus Evolution*. Narnia, 1(1), p. vev011. doi: 10.1093/ve/vev011.

Cleaveland, S. (1998) 'The growing problem of rabies in Africa', *Transactions of the Royal Society of Tropical Medicine and Hygiene*. Oxford University Press, 92(2), pp. 131–134. doi:

10.1016/S0035-9203(98)90718-0.

Cleaveland, S. *et al.* (2014) ‘The changing landscape of rabies epidemiology and control’, *Onderstepoort Journal of Veterinary Research*. Agricultural Research Council, 81(2). doi: 10.4102/ojvr.v81i2.731.

Cliquet, F., Picard-Meyer, E. and Robardet, E. (2014) ‘Rabies in Europe: What are the risks?’, *Expert Review of Anti-Infective Therapy*. Expert Reviews Ltd., pp. 905–908. doi: 10.1586/14787210.2014.921570.

Cobb, B. D. and Clarkson, J. M. (1994) ‘A simple procedure for optimising the polymerase chain reaction (PCR) using modified taguchi methods’, *Nucleic Acids Research*. Oxford Academic, 22(18), pp. 3801–3805. doi: 10.1093/nar/22.18.3801.

Coertse, J. *et al.* (2020) ‘Lyssaviruses in insectivorous bats, South Africa, 2003–2018’, *Emerging Infectious Diseases*. Centers for Disease Control and Prevention (CDC), pp. 3056–3060. doi: 10.3201/EID2612.203592.

Coertse, J. *et al.* (2021) ‘Lagos Bat Virus, an Under-Reported Rabies-Related Lyssavirus’, *Viruses*. NLM (Medline), p. 576. doi: 10.3390/v13040576.

Coetzee, P. and Nel, L. H. (2007) ‘Emerging epidemic dog rabies in coastal South Africa: A molecular epidemiological analysis’, *Virus Research*. Elsevier, 126(1–2), pp. 186–195. doi: 10.1016/J.VIRUSRES.2007.02.020.

Coetzer, A., Anahory, I., *et al.* (2017) ‘Enhanced diagnosis of rabies and molecular evidence for the transboundary spread of the disease in Mozambique’, *Journal of the South African Veterinary Association*, 88(0), p. 9. doi: 10.4102/jsava.v88i0.1397.

Coetzer, A., Coertse, J., *et al.* (2017) ‘Epidemiology of Rabies in Lesotho: The Importance of Routine Surveillance and Virus Characterization’, *Tropical Medicine and Infectious Disease* . doi: 10.3390/tropicalmed2030030.

Coetzer, A. *et al.* (2019) ‘Epidemiological aspects of the persistent transmission of rabies during an outbreak (2010 – 2017) in Harare, Zimbabwe’, *PLOS ONE*. Edited by A. M. Samy, 14(1), p. e0210018. doi: 10.1371/journal.pone.0210018.

Cohen, C., Sartorius, B., Sabeta, C., Zulu, G., Paweska, J., Mogoswane, M., Sutton, C., Nel, Louis H, *et al.* (2007) ‘Epidemiology and molecular virus characterization of reemerging rabies, South Africa.’, *Emerging Infectious Diseases*. Centers for Disease Control and Prevention, 13(12), pp. 1879–86. doi: 10.3201/eid1312.070836.

Cohen, C., Sartorius, B., Sabeta, C., Zulu, G., Paweska, J., Mogoswane, M., Sutton, C., Nel,

Louis H., *et al.* (2007) ‘Epidemiology and molecular virus characterization of reemerging rabies, South Africa’, *Emerging Infectious Diseases*. Centers for Disease Control and Prevention (CDC), 13(12), pp. 1879–1886. doi: 10.3201/eid1312.070836.

Colombi, D. *et al.* (2020) ‘Long-range movements coupled with heterogeneous incubation period sustain dog rabies at the national scale in Africa’, *PLoS Neglected Tropical Diseases*. Public Library of Science, 14(5), p. e0008317.

Courtin, F. *et al.* (2000) ‘Temporal patterns of domestic and wildlife rabies in central Namibia stock-ranching area, 1986-1996’, *Preventive Veterinary Medicine*, 43(1), pp. 13–28. doi: 10.1016/S0167-5877(99)00083-5.

Czupryna, A. M. *et al.* (2016) ‘Ecology and Demography of Free-Roaming Domestic Dogs in Rural Villages near Serengeti National Park in Tanzania’, *PLOS ONE*. Edited by B. L. Allen. Public Library of Science, 11(11), p. e0167092. doi: 10.1371/journal.pone.0167092.

Davis, P. L. *et al.* (2007) ‘The evolutionary dynamics of canid and mongoose rabies virus in southern Africa’, *Archives of Virology*. Springer-Verlag, 152(7), pp. 1251–1258. doi: 10.1007/s00705-007-0962-9.

Drummond, A. J. *et al.* (2012) ‘Bayesian Phylogenetics with BEAUti and the BEAST 1.7’, *Molecular Biology and Evolution*, 29(8), pp. 1969–1973. doi: 10.1093/molbev/mss075.

EWT (no date) *Mammal Red List – The Endangered Wildlife Trust*. Available at: https://www.ewt.org.za/resources/resources-mammal-red-list/mammal-red-list/ (Accessed: 17 February 2020).

Eybpoosh, S. *et al.* (2017) ‘Molecular epidemiology of infectious diseases.’, *Electronic Physician*. The Electronic Physician, 9(8), pp. 5149–5158. doi: 10.19082/5149.

Fahrion, A. S. *et al.* (2017) ‘The Road to Dog Rabies Control and Elimination—What Keeps Us from Moving Faster?’, *Frontiers in Public Health*. Frontiers, 5, p. 103. doi: 10.3389/fpubh.2017.00103.

Fairbanks, D. H. K. *et al.* (2000) ‘The South African Land-cover Characteristics Database: a synopsis of the landscape’, *Research Article South African Journal of Science*, 96. doi: 10.10520/AJA00382353_7429.

Feng, Y. *et al.* (2016) ‘Livestock rabies outbreaks in Shanxi province, China’, *Archives of Virology*. Springer-Verlag Wien, 161(10), pp. 2851–2854. doi: 10.1007/s00705-016-2982-9.

Finnegan, C. J. *et al.* (2002) ‘Rabies in North America and Europe’, *Journal of the Royal Society of Medicine*. SAGE Publications, 95(1), pp. 9–13. doi: 10.1177/014107680209500104.

Freuling, C. M. *et al.* (2013) ‘The elimination of fox rabies from Europe: Determinants of success and lessons for the future’, *Philosophical Transactions of the Royal Society B: Biological Sciences*. The Royal Society, 368(1623). doi: 10.1098/rstb.2012.0142.

Gilbert, A. T. and Chipman, R. B. (2020) ‘Rabies control in wild carnivores’, in *Rabies*. Elsevier, pp. 605–654. doi: 10.1016/b978-0-12-818705-0.00019-4.

Global Alliance for Rabies Control (no date a) *DHIS2 | Global Alliance for Rabies Control*. Available at: https://rabiesalliance.org/dhis2/malawi (Accessed: 17 April 2020).

Global Alliance for Rabies Control (no date b) *DHIS2 | Global Alliance for Rabies Control*. Available at: https://rabiesalliance.org/dhis2/mozambique (Accessed: 17 April 2020).

Global Alliance for Rabies Control (no date c) *DHIS2 | Global Alliance for Rabies Control*. Available at: https://rabiesalliance.org/dhis2/zimbabwe (Accessed: 17 April 2020).

Global Alliance for Rabies Control (no date d) *DHIS2 | Global Alliance for Rabies Control*. Available at: https://rabiesalliance.org/dhis2/lesotho (Accessed: 17 April 2020).

Global Alliance for Rabies Control (no date e) *DHIS2 | Global Alliance for Rabies Control*. Available at: https://rabiesalliance.org/dhis2/eswatini (Accessed: 17 April 2020).

Grewar, J. (no date) *Rabies events in the Western Cape Province 2010 | Agriprobe*. Available at: https://journals.co.za/doi/10.10520/EJC19017 (Accessed: 26 March 2021).

Grobbelaar, A. A. *et al.* (2020) ‘Human rabies associated with domestic cat exposures in South Africa, 1983-2018’, *Journal of the South African Veterinary Association*. AOSIS (pty) Ltd, 91, pp. 1019–9128. doi: 10.4102/jsava.v91i0.2036.

Gummow, B., Roefs, Y. A. A. and de Klerk, G. (2010) ‘Rabies in South Africa between 1993 and 2005: what has been achieved? ’, *Journal of the South African Veterinary Association* . scieloza , pp. 16–21.

Hampson, K. *et al.* (2015) ‘Estimating the Global Burden of Endemic Canine Rabies’, *PLOS Neglected Tropical Diseases*. Edited by M. S. Carvalho, 9(4), p. e0003709. doi: 10.1371/journal.pntd.0003709.

Hassel, R. H. (1982) ‘Incidence of rabies in kudu in South West Africa Namibia’, *South African Journal of Science*. Academy of Science for South Africa (ASSAf), 78(10), p. 418.

Hayman, D. T. S. *et al.* (2011) ‘Evolutionary History of Rabies in Ghana’, *PLoS Neglected Tropical Diseases*. Edited by J. Zinsstag. Public Library of Science, 5(4), p. e1001. doi: 10.1371/journal.pntd.0001001.

Hergert, M., Le Roux, K. and Nel, L. H. (2018) 'Characteristics of owned dogs in rabies endemic KwaZulu-Natal province, South Africa', *BMC Veterinary Research*. BioMed Central Ltd., 14(1), pp. 1–10. doi: 10.1186/s12917-018-1604-z.

Holmala, K. and Kauhala, K. (2006) 'Ecology of wildlife rabies in Europe', *Mammal Review*, 36(1), pp. 17–36. doi: 10.1111/j.1365-2907.2006.00078.x.

Hübschle, O. J. B. (1988) 'Rabies in the Kudu Antelope (Tragelaphus strepsiceros)', *Clinical Infectious Diseases*, 10(Supplement_4), pp. S629–S633. doi: 10.1093/clinids/10.Supplement_4.S629.

Hughes, J. and Macdonald, D. W. (2013) 'A review of the interactions between free-roaming domestic dogs and wildlife', *Biological Conservation*. Elsevier, 157, pp. 341–351. doi: 10.1016/J.BIOCON.2012.07.005.

Hutcheon, D. (1894) 'Reports for the Colonial Veterinary Surgeon for the year 1893', *Cape of Good Hope, Department of Agriculture*, pp. 7–10.

Johnson, N. *et al.* (2004) 'Molecular epidemiology of rabies in Botswana: a comparison between antibody typing and nucleotide sequence phylogeny', *Veterinary Microbiology*. Elsevier, 101(1), pp. 31–38.

King, A. A. *et al.* (2004) *Historical perspective of rabies in Europe and the Mediterranean Basin*. OIE Paris.

Kissi, B., Tordo, N. and Bourhy, H. (1995) 'Genetic Polymorphism in the Rabies Virus Nucleoprotein Gene', *Virology*. Academic Press, 209(2), pp. 526–537. doi: 10.1006/VIRO.1995.1285.

Knobel, D. L. *et al.* (2005) 'Re-evaluating the burden of rabies in Africa and Asia', *Bulletin of the World Health Organization*. World Health Organization, 83, pp. 360–368. doi: 10.1590/S0042-96862005000500012.

Kobayashi, Y. *et al.* (2007) 'Genetic Analysis of Phosphoprotein and Matrix Protein of Rabies Viruses Isolated in Brazil', *Journal of Veterinary Medical Science*. JAPANESE SOCIETY OF VETERINARY SCIENCE, 69(11), pp. 1145–1154. doi: 10.1292/jvms.69.1145.

Köchlin, J. R. (1835) *Ueber die in unsern Zeiten unter den Füchsen herrschende Krankheit und die Natur und Ursachen der Wuthkrankheit überhaupt*. Orell, Füssli & Cie.

Koeppel, K. N., van Schalkwyk, O. L. and Thompson, P. N. (2021) 'Patterns of rabies cases in South Africa between 1993 and 2019, including the role of wildlife', *Transboundary and Emerging Diseases*. Wiley Online Library.

KwaZulu-Natal Department of the Agriculture Rural Development (KZNDARD) (2015) *Jackal rabies in the KwaZulu-Natal Midlands*. Available at: https://www.kzndard.gov.za/images/Documents/AnimalHealth/AnimalDiseases/Jackal-Rabies-in-the-KwaZulu-Natal-Midlands.pdf (Accessed: 25 May 2021).

Lembo, T. *et al.* (2008) 'Exploring reservoir dynamics: A case study of rabies in the Serengeti ecosystem', *Journal of Applied Ecology*. John Wiley & Sons, Ltd, 45(4), pp. 1246–1257. doi: 10.1111/j.1365-2664.2008.01468.x.

Lembo, T. *et al.* (2010) 'The Feasibility of Canine Rabies Elimination in Africa: Dispelling Doubts with Data', *PLoS Neglected Tropical Diseases*. Edited by C. E. Rupprecht. Public Library of Science, 4(2), p. e626. doi: 10.1371/journal.pntd.0000626.

LeRoux, K. *et al.* (2018) 'Rabies control in Kwazulu-Natal, South Africa', *Bulletin of the World Health Organization*. World Health Organization, 96(5), pp. 360–365. doi: 10.2471/BLT.17.194886.

Magembe, S. R. (1985) 'Epidemiology of rabies in the United Republic of Tanzania', in *Rabies in the Tropics*. Springer, pp. 392–398.

Mähl, P. *et al.* (2014) 'Twenty year experience of the oral rabies vaccine SAG2 in wildlife: A global review', *Veterinary Research*. BioMed Central Ltd., pp. 1–17. doi: 10.1186/s13567-014-0077-8.

Mallewa, M. *et al.* (2007) 'Rabies encephalitis in malaria-endemic area, Malawi, Africa', *Emerging Infectious Diseases*. Centers for Disease Control and Prevention, 13(1), p. 136.

Mansvelt, P. R. (1962) 'Rabies in South Africa: field control of the disease', *Journal of the South African Veterinary Association*. AOSIS, 33(3), pp. 313–319.

Markotter, W. *et al.* (2006) 'Isolation of Lagos bat virus from water mongoose', *Emerging Infectious Diseases*. Centers for Disease Control and Prevention (CDC), 12(12), pp. 1913–1918. doi: 10.3201/eid1212.060514.

Mkhize, G. C. *et al.* (2010) 'Re-Emergence of Dog Rabies in Mpumalanga Province, South Africa', *Vector-Borne and Zoonotic Diseases*, 10(9), pp. 921–926. doi: 10.1089/vbz.2009.0109.

Müller, T. *et al.* (2012) 'Elimination of terrestrial rabies in Germany using oral vaccination of foxes', *Berl Munch Tierarztl Wochenschr*, 125(5–6), pp. 178–190.

Müller, W., Cox, J. and Müller, T. (2004) 'Rabies in Germany, Denmark and Austria', *Historical perspectives of rabies in Europe and the Mediterranean basin*. OIE, 79, p. 92.

Munang'andu, H. M. *et al.* (2011) 'Rabies Status in Zambia for the Period 1985-2004', *Zoonoses and Public Health*. John Wiley & Sons, Ltd, 58(1), pp. 21–27. doi: 10.1111/j.1863-2378.2010.01368.x.

Municipalities of South Africa (2021) *Limpopo Municipalities*. Available at: https://municipalities.co.za/provinces/view/5/limpopo (Accessed: 26 August 2021).

Nadin-Davis, S. A. *et al.* (2000) 'A panel of monoclonal antibodies targeting the rabies virus phosphoprotein identifies a highly variable epitope of value for sensitive strain discrimination', *Journal of Clinical Microbiology*. American Society for Microbiology, 38(4), pp. 1397–1403. doi: 10.1128/jcm.38.4.1397-1403.2000.

Nel, L. H. *et al.* (2005) 'Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology', *Virus Research*. Elsevier, 109(2), pp. 165–173. doi: 10.1016/J.VIRUSRES.2004.12.003.

Nel, L. H. (2013) 'Discrepancies in data reporting for rabies, Africa.', *Emerging infectious diseases*. Centers for Disease Control and Prevention, 19(4), pp. 529–33. doi: 10.3201/eid1904.120185.

Nel, L. H. and Rupprecht, C. E. (2007a) 'Emergence of lyssaviruses in the old world: The case of Africa', *Current Topics in Microbiology and Immunology*. Springer, Berlin, Heidelberg, pp. 161–193. doi: 10.1007/978-3-540-70962-6_8.

Nel, L. H. and Rupprecht, C. E. (2007b) 'Emergence of Lyssaviruses in the Old World: The Case of Africa', in. Springer, Berlin, Heidelberg, pp. 161–193. doi: 10.1007/978-3-540-70962-6_8.

Ngoepe, C. E., Sabeta, C. and Nel, L. (2009) 'The spread of canine rabies into Free State province of South Africa: A molecular epidemiological characterization', *Virus Research*. Elsevier, 142(1–2), pp. 175–180. doi: 10.1016/j.virusres.2009.02.012.

Van Niekerk, H. N. (2010) 'The cost of predation on small livestock in South Africa by medium-sized predators'. University of the Free State.

North West Municipalities (no date). Available at: https://municipalities.co.za/provinces/view/8/north-west (Accessed: 26 August 2021).

OIE (no date) *Self-declared disease status : OIE - World Organisation for Animal Health*. Available at: https://www.oie.int/animal-health-in-the-world/self-declared-disease-status/ (Accessed: 19 February 2020).

Pasteur, L. (1887) *Hydrophobia : an account of M. Pasteur's system : containing a translation*

of all his communications on the subject, the technique of his method, and the latest statistical results. 1st edn. Edited by J. R. Suzor. London.

Picard-Meyer, E. *et al.* (2011) 'Active surveillance of bat rabies in France: A 5-year study (2004-2009)', *Veterinary Microbiology*. Elsevier, 151(3–4), pp. 390–395. doi: 10.1016/j.vetmic.2011.03.034.

Rupprecht, C. E. *et al.* (1995) 'The ascension of wildlife rabies: A cause for public health concern or intervention?', *Emerging Infectious Diseases*. Centers for Disease Control and Prevention (CDC), 1(4), pp. 107–114. doi: 10.3201/eid0104.950401.

Sabeta, C. T. *et al.* (2007) 'Molecular epidemiology of rabies in bat-eared foxes (Otocyon megalotis) in South Africa', *Virus Research*, 129(1), pp. 1–10. doi: 10.1016/j.virusres.2007.04.024.

Sabeta, C.T. *et al.* (2007) 'Molecular epidemiology of rabies in bat-eared foxes (Otocyon megalotis) in South Africa', *Virus Research*. Elsevier, 129(1), pp. 1–10. doi: 10.1016/J.VIRUSRES.2007.04.024.

Sabeta, C. T. *et al.* (2013) 'Emergence of rabies in the Gauteng Province, South Africa: 2010-2011', *Journal of the South African Veterinary Association*. AOSIS Publishing, 84(1), p. 0.

Sabeta, C. T. *et al.* (2020) 'Rabies in the African Civet: An Incidental Host for Lyssaviruses?', *Viruses*. Multidisciplinary Digital Publishing Institute, 12(4), p. 368. doi: 10.3390/v12040368.

Sabeta, C. T., Bingham, J. and Nel, L. H. (2003) 'Molecular epidemiology of canid rabies in Zimbabwe and South Africa', *Virus Research*. Elsevier, 91(2), pp. 203–211. doi: 10.1016/S0168-1702(02)00272-1.

Sabeta, C. T., Mkhize, G. C. and Ngoepe, E. C. (2011a) 'An evaluation of dog rabies control in Limpopo province (South Africa)', *Epidemiology and Infection*, 139(10), pp. 1470–1475. doi: 10.1017/S0950268811001233.

Sabeta, C. T., Mkhize, G. C. and Ngoepe, E. C. (2011b) 'An evaluation of dog rabies control in Limpopo province (South Africa)', *Epidemiology and Infection*. Cambridge University Press, 139(10), pp. 1470–1475. doi: 10.1017/S0950268811001233.

Sacramento, D. *et al.* (1992) 'Molecular epidemiology of rabies virus in France: comparison with vaccine strains', *Journal of General Virology*, 73(5), pp. 1149–1158. doi: 10.1099/0022-1317-73-5-1149.

Sacramento, D., Bourhy, H. and Tordo, N. (1991) 'PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus', *Molecular and Cellular Probes*.

Academic Press, 5(3), pp. 229–240. doi: 10.1016/0890-8508(91)90045-L.

Schneider, H. P. (1985) ‘Rabies in South West Africa/Namibia’, in *Rabies in the Tropics*. Springer, pp. 520–535.

Schneider, L. G. and Cox, H. J. (1983) ‘A field trial for the oral immunization of foxes against rabies in the Federal Republic of Germany, 1: Safety, efficacy and stability of the SAD B19 vaccine.’, *Tieraerztliche Umschau*.

Scott, T. P. *et al.* (2013) ‘Complete Genome and Molecular Epidemiological Data Infer the Maintenance of Rabies among Kudu (Tragelaphus strepsiceros) in Namibia’, *PLoS ONE*. Edited by B. E. Dutilh. Public Library of Science, 8(3), p. e58739. doi: 10.1371/journal.pone.0058739.

Scott, T. P. *et al.* (2017) ‘Addressing the Disconnect between the Estimated, Reported, and True Rabies Data: The Development of a Regional African Rabies Bulletin’, *Frontiers in Veterinary Science*. Frontiers, 4, p. 18. doi: 10.3389/fvets.2017.00018.

Scott, T. P., Coetzer, A. and Nel, L. H. (2016) ‘Rabies in Namibia, more than a horrendous disease; the social, environmental, and economic challenges faced’, *Current Politics and Economics of Africa*. Hauppauge: Nova Science Publishers, Inc., 9(1), pp. 131–167. Available at: https://search.proquest.com/scholarly-journals/rabies-namibia-more-than-horrendous-disease/docview/2007082227/se-2?accountid=14717.

Scott, T. P., Hassel, R. and Nel, L. H. (2012) ‘Rabies in kudu (Tragelaphus strepsiceros)’. Schlütersche Verlagsgesellschaft.

Shite, A., Guadu, T. and Admassu, B. (2015) ‘Challenges of rabies’, *International Journal of Basic and Applied Virology*, 4(2), pp. 41–52.

Shwiff, S. A. *et al.* (2016) ‘Towards Canine Rabies Elimination in KwaZulu-Natal, South Africa: Assessment of Health Economic Data’, *Transboundary and Emerging Diseases*. Blackwell Publishing Ltd, 63(4), pp. 408–415. doi: 10.1111/tbed.12283.

Siongok, T. K. A. and Karama, M. (1985) ‘Epidemiology of human rabies in Kenya’, in *Rabies in the Tropics*. Springer, pp. 445–450.

Van Sittert, S. J. *et al.* (2010) ‘Rabies in the Eastern Cape Province of South Africa-where are we going wrong?’, *Journal of the South African Veterinary Association*. AOSIS Publishing, 81(4), pp. 207–215.

Smith, J. S. *et al.* (1992) ‘Epidemiologic and Historical Relationships among 87 Rabies Virus Isolates as Determined by Limited Sequence Analysis’, *Journal of Infectious Diseases*. Narnia,

166(2), pp. 296–307. doi: 10.1093/infdis/166.2.296.

Smith, J. S. (1996) *New Aspects of Rabies with Emphasis on Epidemiology, Diagnosis, and Prevention of the Disease in the United States*, *CLINICAL MICROBIOLOGY REVIEWS*.

Smith, J. S., Orciari, L. A. and Yager, P. A. (1995) 'Molecular epidemiology of rabies in the united states', *Seminars in Virology*, 6(6), pp. 387–400. doi: 10.1016/S1044-5773(05)80016-2.

Snyman, P. S. (1940) 'The study and control of the vectors of rabies in South Africa'. Pretoria: The Government Printer.

StatsSA (2020a) *Census of Commercial Agriculture*. Available at: http://www.statssa.gov.za/publications/Report-11-02-01/CoCA 2017 Fact Sheets.pdf (Accessed: 9 January 2021).

StatsSA (2020b) *North West Publication | Statistics South Africa*. Available at: http://www.statssa.gov.za/?page_id=1854&PPN=Report-11-02-07&SCH=7908 (Accessed: 9 January 2021).

Swanepoel, R. *et al.* (1993) *Rabies in southern Africa*, *Onderstepoort Journal of Veterinary Research*. Available at: https://pdfs.semanticscholar.org/7781/c90a57d2000a8864b2af198e71ea7b4cebe0.pdf (Accessed: 14 April 2019).

Talbi, C. *et al.* (2009) 'Evolutionary history and dynamics of dog rabies virus in western and central Africa', *Journal of General Virology*. Microbiology Society, 90(4), pp. 783–791. doi: 10.1099/vir.0.007765-0.

Taylor, D. (1976) 'Rabies: epizootic aspects', *Veterinary Record*. British Medical Journal Publishing Group, 99(9), pp. 157–160.

von Teichman, B. F. *et al.* (1995) 'Molecular epidemiology of rabies virus in South Africa: evidence for two distinct virus groups', *Journal of General Virology*, 76(1), pp. 73–82. doi: 10.1099/0022-1317-76-1-73.

Thomson, G. and Meredith, C. (1993) 'Wildlife rabies in southern Africa', *Rabies in Eastern and Southern Africa*.

Tordo, N. and Kouknetzoff, A. (1993) *The rabies virus genome: an overview*, *Onderstepoort Journal of Veterinary Research*. Available at: https://pdfs.semanticscholar.org/3c94/c168619e039d415281d18a7962bfa9f2a0e1.pdf (Accessed: 6 March 2019).

United Nations (no date) *Take Action for the Sustainable Development Goals – United Nations Sustainable Development*. Available at: https://www.un.org/sustainabledevelopment/sustainable-development-goals/ (Accessed: 5 January 2021).

Vaughn, J. B. (1975) 'Cat rabies', *The natural history of rabies*, 2, pp. 139–154.

Velasco-Villa, A. *et al.* (2008) 'Enzootic rabies elimination from dogs and reemergence in wild terrestrial carnivores, United States', *Emerging Infectious Diseases*. Centers for Disease Control and Prevention, 14(12), pp. 1849–1854. doi: 10.3201/eid1412.080876.

Vigilato, M. A. N. *et al.* (2013) 'Progress towards eliminating canine rabies: policies and perspectives from Latin America and the Caribbean', *Philosophical Transactions of the Royal Society B: Biological Sciences*. The Royal Society, 368(1623), pp. 20120143–20120143. doi: 10.1098/rstb.2012.0143.

Vos, A. *et al.* (2014) 'Bovine rabies in Turkey: Patterns of infection and implications for costs and control', *Epidemiology and Infection*. Cambridge University Press, 142(9), pp. 1925–1933. doi: 10.1017/S0950268813002811.

Wachendorfer, G. *et al.* (1986) 'Erfahrungen mit der oralen Immunisierung von Fuchsen gegen Tollwut in Hessen', *Tierharztliche Praxis*.

Wandeler, A. I. (1988) 'Control of Wildlife Rabies: Europe', in. Springer, Boston, MA, pp. 365–380. doi: 10.1007/978-1-4613-1755-5_16.

Westerling, B. *et al.* (2004) 'Rabies in the Baltics', *Historical perspective of rabies in Europe and Mediterranean Basin, Paris, OIE Publications*, pp. 33–46.

Weyer, J. *et al.* (2011) 'Epidemiology of human rabies in South Africa, 1983-2007', *Virus Research*. Elsevier, 155(1), pp. 283–290. doi: 10.1016/j.virusres.2010.10.023.

Weyer, J. (2015) 'Rabies in South Africa: where do we stand in 2015?', *Southern African Journal of Infectious Diseases*. AOSIS, 30(2), pp. 40–41.

Wheeler, D. C. and Waller, L. A. (2008) 'Mountains, valleys, and rivers: the transmission of raccoon rabies over a heterogeneous landscape', *Journal of Agricultural, Biological, and Environmental Statistics*. Springer, 13(4), pp. 388–406.

WHO (2018) *WHO expert consultation on rabies: third report*. Available at: https://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf (Accessed: 15 March 2019).

Wolff, F. and Frisch, R. (1985) 'Nineteen years of rabies in Luxembourg', *Annales de*

Medecine Veterinaire (Belgium).

World Health Organization (2017) *WHO | Weekly Epidemiological Record, 17 February 2017, vol. 92, 7 (pp. 77–88)*, *WHO*. World Health Organization. Available at: https://www.who.int/wer/2017/wer9207/en/ (Accessed: 27 March 2019).

Zulu, G. C., Sabeta, C. T. and Nel, L. H. (2008) 'Trans-border Transmission of Rabies Between South Africa, Zimbabwe and Mozambique Resulted in the Re-emergence of Rabies in Northern South Africa', *International Journal of Infectious Diseases*. Elsevier, 12, p. e130. doi: 10.1016/j.ijid.2008.05.323.

Zulu, G. C., Sabeta, C. T. and Nel, L. H. (2009) 'Molecular epidemiology of rabies: Focus on domestic dogs (Canis familiaris) and black-backed jackals (Canis mesomelas) from northern South Africa', *Virus Research*. Elsevier, 140(1–2), pp. 71–78. doi: 10.1016/J.VIRUSRES.2008.11.004.

Van Zyl, N., Markotter, W. and Nel, L. H. (2010) 'Evolutionary history of African mongoose rabies', *Virus Research*. Elsevier, 150(1–2), pp. 93–102. doi: 10.1016/J.VIRUSRES.2010.02.018.

Additional materials

Ethical Clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Natural and Agricultural Sciences
Ethics Committee

E-mail: ethics.nas@up.ac.za

15 July 2020

ETHICS SUBMISSION: LETTER OF APPROVAL

Miss AJ Malan
Department of Biochemistry, Genetics and Microbiology
Faculty of Natural and Agricultural Science
University of Pretoria

Reference number: NAS085/2020
Project title: Investigating the interface of sylvatic and dog rabies in southern Africa

Dear Miss AJ Malan,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Natural and Agricultural Sciences Research Ethics committee.

Please note the following about your ethics approval:

- Please use your reference number (NAS085/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
- Please note that ethical approval is granted for the duration of the research (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
- The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
- **Applications using Animals:** NAS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Post approval submissions including application for ethics extension and amendments to the approved application should be submitted online via the Ethics work centre.

We wish you the best with your research.

Yours sincerely,

Chairperson: NAS Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

7 September 2020

Approval Certificate
New Application

AEC Reference No.: NAS085/2020
Title: Investigating the interface of sylvatic and dog rabies in southern Africa
Researcher: Miss AJ Malan
Student's Supervisor: Prof LH Nel

Dear Miss AJ Malan,

The **New Application** as supported by documents received between 2020-08-11 and 2020-09-04 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-09-04.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Cattle (Bos Taurus)	53 (post mortem samples)
Cats (Feline)	1 (post mortem samples)
Dogs (Domestic dog)	101 (post mortem samples)
Goats (Caprine)	6 (post mortem samples)
Sheep (Ovine)	3 (post mortem samples)
Genet	2 (post mortem samples)
Aardwolf	3 (post mortem samples)
Bat-eared fox	3 (post mortem samples)
Mongoose	6 (post mortem samples)
Jackal	37 (post mortem samples)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-09-07.

3. Please remember to use your protocol number (NAS085/2020) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.

6. As part of your approval, the committee requires that you record a **short video footage** of major animal procedures approved in your study. The committee may request them for monitoring purposes at any later point.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakediruiwa

Ethics approval is subject to the following:

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

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Tables

Table A1: Species involved in the epidemiology of rabies in South Africa between 1998 and 2019

Species (Scientific name)	Number of samples tested between 1998 and 2019	Number of positive samples	% Positive
Aardvark (*Orycteropus afer*)	1	1	100
Aardwolf (*Proteles cristata*)	113	76	59.3
African civet (*Civettictis civetta*)	33	12	35.3
African clawless otter (*Aonyx capensis*)	11	3	27.3
African palm civet (*Nandinia binotata*)	1	0	0.00
African pygmy mouse (*Mus minutoides*)	1	0	0.00
African striped weasel *(Poecilogale albinucha*)	34	1	2.94
African wild dog (*Lycaon pictus*)	42	10	23.8
African wildcat (*Felis lybica*)	68	36	52.9
African yellow bat (*Scotophilus dinganii*)	51	0	0.00
Angolan free-tailed bat (*Mops condylurus*)	9	0	0.00
Ansorge's free-tailed bat (*Chaerephon ansorgei*)	2	0	0.00
Banana pipistrelle (*Neoromicia nana*)	15	0	0.00
Banded mongoose (*Mungos mungo*)	56	4	7.10
Bat-eared fox (*Otocyon megalotis*)	470	344	73.2
Bay duiker (*Cephalophus dorsalis*)	1	1	100
Black rat (*Rattus rattus*)	3	0	0.00
Black rhinoceros (*Diceros bicornis*)	1	1	100
Black wildebeest (*Connochaetes gnou*)	2	1	50.0
Black-backed jackal (*Canis mesomelas*)	466	254	54.5
Black-footed cat (*Felis nigripes*)	6	4	66.7
Black-tailed tree rat (*Thallomys nigricauda*)	1	0	0.00
Blasius's horseshoe bat (*Rhinolophus blasii*)	12	0	0.00
Blesbok (*Damaliscus dorcas phillipsi*)	4	0	0.00
Blue duiker (*Philantomba monticola*)	1	0	0.00
Blue monkey (*Cercopithecus mitis*)	3	0	0.00

Blue wildebeest (*Connochaetes taurinus*)	12	1	8.30
Bontebok (*Damaliscus pygargus phillipsi*)	6	0	0.00
Botswanan long-eared bat (*Laephotis botswanae*)	2	0	0.00
Bovine (*Bos taurus*)	5,043	2,026	40.2
Brown fur seal (*Arctocephalus pusillus*)	7	0	0.00
Brown hyena (*Hyeana brunnea*)	3	0	0.00
Brown rat (*Rattus norvegicus*)	20	0	0.00
Burchell's zebra (*Equus quagga burchelli*)	3	0	0.00
Bushpig (*Potamochoerus larvatus*)	5	0	0.00
Bush rat (*Rattus* spp.)	1	0	0.00
Bush vlei rat (*Otomys unisulcatis*)	2	0	0.00
Bushveld horseshoe bat (*Rhinolophus simulator*)	50	0	0.00
Cane rat (*Thryonomys* spp.)	4	0	0.00
Cape buffalo (*Syncerus caffer*)	33	1	3.03
Cape bushbuck (*Tragelaphus sylvaticus*)	26	1	3.85
Cape fox (*Vulpes chama*)	34	12	35.3
Cape genet (*Genetta tigrina*)	7	0	0.00
Cape gray mongoose (*Galerella pulverulenta*)	66	19	29.2
Cape ground squirrel (*Xerus inauris*)	117	18	15.4
Cape hairy bat (*Myotis tricolor*)	11	0	0.00
Cape hare (*Lepus capensis*)	1	0	0.00
Cape porcupine (*Hystrix africaeaustralis*)	10	0	0.00
Cape serotine (*Neoromicia capensis*)	69	0	0.00
Caprine (*Capra* spp.)	533	344	64.7
Capybara (*Hydrochoerus hydrochaeris*)	1	0	0.00
Caracal (*Caracal caracal*)	41	14	34.1
Chacma baboon (*Papio ursinus*)	60	2	3.33
Cheetah (*Acinonyx jubatus*)	7	0	0.00
Colombian red howler (*Alouatta seniculus*)	2	0	0.00
Common bent-wing bat (*Miniopterus schreibersii*)	8	0	0.00
Common duiker (*Sylvicapra grimmia*)	35	9	25.7

Common dwarf mongoose (*Helogale parvula*)	9	0	0.00
Common eland (*Taurotragus oryx*)	25	9	36.0
Common marmoset (*Callithrix jacchus*)	1	0	0.00
Common warthog (*Phacochoerus africanus*)	14	0	0.00
Congo rope squirrel (*Funisciurus congicus*)	1	1	100
Damara horseshoe bat (*Rhinolophus damarensis*)	5	0	0.00
Damara woolly bat (*Kerivoula argentata*)	1	0	0.00
Darling's horseshoe bat (*Rhinolophus darlingii*)	5	0	0.00
Dassie rat (*Petromus typicus*)	2	0	0.00
Dent's horseshoe bat (*Rhinolophus denti*)	5	0	0.00
Desert warthog (*Phacochoerus aethiopicus*)	6	1	16.7
Domestic dog (*Canis lupus familiaris*)	19,044	6,719	35.3
Domestic water buffalo (*Bubalus bubalis*)	1	0	0.00
Donkey (*Equus asinus*)	22	12	54.5
Dusky pipistrelle (*Pipistrellus hesperidus*)	20	0	0.00
Eastern gray squirrel (*Sciurus carolinensis*)	4	0	0.00
Egyptian free-tailed bat (*Tadarida aegyptiaca*)	17	0	0.00
Egyptian mongoose (*Herpestes ichneumon*)	11	0	0.00
Egyptian slit-faced bat (*Nycteris thebaica*)	42	1	2.40
Elephant (*Loxodonta africana*)	3	0	0.00
Equine (*Equus* spp.)	271	69	25.5
European hamster (*Cricetus cricetus*)	1	0	0.00
European polecat (*Mustela putorius*)	2	0	0.00
European rabbit (*Oryctolagus cuniculus*)	6	0	0.00
Feline (*Felis catus*)	3,409	333	9.77
Forest shrew (*Myosorex varius*)	1	0	0.00
Four-striped grass mouse (*Rhabdomys pumilio*)	2	0	0.00
Gemsbok (*Oryx gazella*)	3	0	0.00
Genet (*Genetta genetta*)	276	31	11.2
Genet spp. (*Genetta* spp.)	1	0	0.00

Geoffroy's horseshoe bat (*Rhinolophus clivosus*)	30	0	0.00
Giraffe (*Giraffa camelopardis*)	12	0	0.00
Golden wildebeest (*Connochaetes taurinus*)	1	0	0.00
Gray climbing mouse (*Dendromus melanotis*)	1	0	0.00
Greater cane rat (*Thryonomys swinderianus*)	10	0	0.00
Greater kudu (*Tragelaphus strepsiceros*)	111	5	4.50
Greenish yellow bat (*Scotophilus viridus*)	7	0	0.00
Grey rhebok (*Pelea capreolus*)	1	0	0.00
Grivet (*Chlorocebus aethiops*)	4	0	0.00
Guinea pig (*Cavia porcellus*)	2	0	0.00
Hamster (Rodentia spp.)	12	0	0.00
Hartebeest (*Alcelaphus buselaphus*)	1	0	0.00
Heller's pipistrelle (*Neoromicia helios*)	6	0	0.00
Hewitt's red rock hare (*Pronolagus saundersiae*)	1	0	0.00
Hildebrandt's horseshoe bat (*Rhinolophus hilderbrandtii*)	3	0	0.00
Honey badger (*Mellivora capensis*)	32	16	50.0
House mouse (*Mus musculus*)	5	0	0.00
Impala (*Aepyceros melampus*)	25	0	0.00
Jameson's red rock hare (*Pronolagus randensis*)	1	0	0.00
Kuhl's pipistrelle (*Pipistrellus kuhlii*)	2	0	0.00
Lander's horseshoe bat (*Rhinolophus landeri*)	1	0	0.00
Large-eared free-tailed bat (*Otomops martiensseni*)	8	0	0.00
Leopard (*Panthera pardus*)	51	1	1.96
Lesser bushbaby (*Galago moholi*)	16	0	0.00
Lesser cane rat (*Thryonomys gregorianus*)	3	0	0.00
Lesser long-fingered bat (*Miniopterus fraterculus*)	4	0	0.00
Lion (*Panthera leo*)	87	3	3.45
Little free-tailed bat (*Chaerephon pumilus*)	17	0	0.00

Long-tailed house bat (*Eptesicus hottentotus*)	1	0	0.00
Marmoset (*Callithrix* spp.)	1	0	0.00
Marsh mongoose (*Atilax paludinosis*)	109	20	18.2
Mauritian tomb bat (*Taphozous mauritianus*)	3	0	0.00
Meerkat (*Suricata suricatta*)	557	107	19.2
Midas free-tailed bat (*Mops midas*)	3	0	0.00
Mole (Unknown spp.)	6	1	16.7
Mountain ground squirrel (*Xerus princeps*)	3	0	0.00
Namaqua rock rat (*Aethomys namaquensis*)	13	0	0.00
Natal long-fingered bat (*Miniopterus natalensis*)	125	3	2.40
Nyala (*Tragelaphus angasii*)	21	0	0.00
Ovine (*Ovis aries*)	403	187	46.1
Percival's trident bat (*Cloeotis percivali*)	5	0	0.00
Porcine (*Sus domesticus*)	107	22	20.8
Raccoon (*Procyon lotor*)	2	0	0.00
Red forest duiker (*Cephalophus natalensis*)	1	0	0.00
Red river hog (*Potamochoerus porcus*)	1	0	0.00
Red rock rat (*Aethomys crysophilus*)	1	0	0.00
Rendall's serotine (*Neoromicia rendalii*)	1	0	0.00
Roan (*Hippotragus equinus*)	2	0	0.00
Robert's flat-headed bat (*Sauromys petrophilus*)	1	0	0.00
Rock hyrax (*Procavia capensis*)	203	3	1.48
Rusty pipistrelle (*Pipistrellus rusticus*)	35	0	0.00
Rusty-spotted genet (*Genetta maculata*)	1	0	0.00
Sable (*Hippotragus niger*)	22	0	0.00
Schlieffen's bat (*Nycticeinops schlieffeni*)	8	0	0.00
Scrub hare (*Lepus saxatilis*)	1	0	0.00
Selous's mongoose (*Paracynictis selousi*)	1	1	100
Senegal bushbaby (*Galago senegalensis*)	1	0	0.00
Serval (*Leptailurus serval*)	23	4	17.4
Sharpe's grysbok (*Raphicerus sharpei*)	1	0	0.00
Shrew (*Crocidura* spp.)	7	0	0.00

Side-striped jackal (*Canis adustus*)	15	4	26.7
Slender mongoose (*Galerella sanguinea*)	383	99	25.8
Smith's bush squirrel (*Paraxerus cepapi*)	18	0	0.00
Smithers's horseshoe bat (*Rhinolophus smithersi*)	4	0	0.00
Somali serotine (*Neoromicia somalica*)	1	0	0.00
South African pouched mouse (*Saccostomus campestris*)	1	0	0.00
South African springhare (*Pedestes capensis*)	27	0	0.00
South African vlei rat (*Otomys irroratus*)	2	0	0.00
Southern multimammute mouse (*Mastomys coucha*)	1	0	0.00
Southern reedbuck (*Redunca arundinum*)	7	0	0.00
Spotted hyena (*Crocuta crocuta*)	35	3	8.57
Spotted-necked otter (*Hydrictis maculicollis*)	1	0	0.00
Springbok (*Antidorcas marsupialis*)	21	0	0.00
Squirrel spp. (*Sciuridae* spp.)	5	0	0.00
Steenbok (*Raphicerus campestris*)	17	0	0.00
Striped polecat (*Ictonyx striatus*)	236	13	5.51
Sundevall's roundleaf bat (*Hipposideros caffer*)	17	0	0.00
Swinny's horseshoe bat (*Rhinolophus swinnyi*)	3	0	0.00
Tapir spp. (*Tapirus* spp.)	1	0	0.00
Tiger (*Panthera tigris*)	2	0	0.00
Unknown antelope spp.	5	0	0.00
Unknown ape spp.	3	0	0.00
Unknown bat spp.	192	6	3.13
Unknown beaver spp.	1	0	0.00
Unknown bushbaby spp. (*Galago* spp.)	16	0	0.00
Unknown camel spp. (*Camelus* spp.)	1	0	0.00
Unknown cane rat (*Thryonomys* spp.)	1	0	0.00
Unknown Canis spp.	6	1	16.7

Unknown deer spp.	3	1	33.3
Unknown duiker spp.	17	1	5.88
Unknown fox spp.	11	4	36.4
Unknown genet spp. (*Genetta* spp.)	17	0	0.00
Unknown hamster spp. (*Cricetinae* spp.)	8	0	0.00
Unknown hyena spp. (*Hyaenidae* spp.)	25	4	16.0
Unknown jackal spp. (*Canis* spp.)	514	215	41.8
Unknown mongoose spp.	404	67	16.6
Unknown mongoose spp. (*Galerella* spp.)	1	1	100
Unknown mongoose spp. (*Herpestidae* spp.)	874	159	18.1
Unknown monkey spp.	128	0	0.00
Unknown mouse spp. (*Rhabdomys* spp)	75	0	0.00
Unknown mouse spp.	24	0	0.00
Unknown panthera spp. (*Panthera* spp)	1	0	0.00
Unknown rabbit spp. (*Lagomorpha* spp)	55	0	0.00
Unknown rat spp. (*Rattus* spp.)	190	0	0.00
Unknown rhino spp.	2	0	0.00
Unknown rodent spp. (*Rodentia* spp.)	91	0	0.00
Unknown shrew spp. (*Soricidae* spp.)	7	0	0.00
Unknown squirrel spp.	40	0	0.00
Unknown weasel spp.	1	0	0.00
Unknown wild pig	1	0	0.00
Unknown wildebeest spp. (*Connochaetes* spp.)	6	1	16.7
Unknown wildlife	184	30	16.3
Variegated butterfly bat (*Glauconycteris variegate*)	4	0	0.00
Vervet monkey (*Cercopithecus aethiops*)	22	0	0.00
Vervet monkey (*Chlorocebus pygerythrus*)	24	1	4.20
Wahlberg's epauletted fruit bat (*Epomophorus wahlbergi*)	31	4	12.9
Waterbuck (*Kobus ellipsiprymnus*)	10	0	0.00
Welwitsch’s bat (*Myotis welwitschia*)	1	0	0.00
White rhinoceros (*Ceratotherium simum*)	2	0	0.00

White-bellied yellow bat (*Scotophilus leucogaster*)	5	0	0.00
White-tailed mongoose (*Ichneumia albicauda*)	18	1	5.56
White-tailed rat (*Mystromys albicaudatus*)	1	0	0.00
Wild boar (*Sus scrofa*)	1	0	0.00
Wildebeest (*Connochaetes*)	9	0	0.00
Woosnam's broad-headed mouse (*Zelotomys woosnami*)	1	0	0.00
Yellow mongoose (*Cynictis penicillata*)	1,275	628	49.3
Zebra spp. (*Equus* spp.)	14	2	14.3
Zulu serotine (*Neoromicia zuluensis*)	3	0	0.00
Total	38,081	11,989	**31.5**

*For some samples, the province of origin was unknown and were subsequently excluded from the analysis. The number of samples indicated in this table show the total number of samples per species across South Africa (irrespective of the province of origin).

Table A2: List of RABV sequences included in the phylogenetic analysis for partial N gene in the NW province. Samples numbers from this study are shown in bold.

Year Sampled	Sample Number	Sequenced	Species	Country	Province	Latitude	Longitude	Accession number
1989	399	Previously published	Jackal	Botswana	Tshabong	-25.754	22.41838	AY330747
1990	420/90	Previously published	Yellow mongoose	South Africa	North West	-27.1974	25.98311	FJ392383
1991	19671	Previously published	African civet	Zimbabwe	Rusape	-18.5279	32.12843	KY553266
1994	22574	Previously published	African civet	Zimbabwe	Wedza	-18.6173	31.5736	KY553255
2006	696/06	Previously published	Yellow mongoose	South Africa	Free State	-27.6504	27.23488	JQ692994
2008	UPV128	Previously published	Canine	South Africa	KwaZulu-Natal	-29.8579	31.0292	JF747613
2011	11_185	Previously published	Canine	South Africa	KwaZulu-Natal	-29.5093	30.19838	KJ744305
2012	555/12	Previously published	Hyena	South Africa	North West	-25.2775	27.21605	KT892004
2012	556/12	Previously published	Canine	South Africa	North West	-25.2775	27.21605	KT892003
2012	566/12	Previously published	Black-backed jackal	South Africa	North West	-25.2775	27.21605	KT892002
2014	889/14	Previously published	African wild dog	South Africa	North West	-24.7435	26.25732	KT891999
2015	113/15	Previously published	Hyena	South Africa	North West	-24.7435	26.25732	KT891998
2015	471/15	Previously published	Canine	South Africa	North West	-27.2371	26.23514	MT454634
2015	682/15	Previously published	Canine	South Africa	North West	-24.7435	26.25732	MT454635
2016	516/16	Previously published	Canine	South Africa	North West	-25.4261	27.2243	MT454636

2016	635/16	Previously published	Canine	South Africa	North West	-25.8026	27.87506	MT454639
2017	**NWdog17/17**	This study	Canine	South Africa	North West	-25.7905	27.2421	MW344002
2017	**NWbov22/17**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW344003
2017	**NWdog31/17**	This study	Canine	South Africa	North West	-25.1609	27.16296	MW344014
2017	**NWbov57/17**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW344005
2017	**NWbov59/17**	This study	Bovine	South Africa	North West	-27.1887	25.32931	MW344007
2017	**NWbov62/17**	This study	Bovine	South Africa	North West	-26.8648	24.79046	MW344015
2017	**NWbov74/17**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW344011
2017	**NWbov126/17**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW344016
2017	**NWjac198/17**	This study	Jackal	South Africa	North West	-26.6864	25.45907	MW344017
2017	**NWbov331/17**	This study	Bovine	South Africa	North West	-27.1887	25.32931	MW344018
2017	**NWbov432/17**	This study	Bovine	South Africa	North West	-26.1739	26.46947	MW343988
2017	**NWbov435/17**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW344004
2017	**NWcap528/17**	This study	Caprine	South Africa	North West	-27.5311	24.78659	MW343987
2017	**NWovi583/17**	This study	Ovine	South Africa	North West	-27.5311	24.78659	MW344006
2017	**NWbov604/17**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW343989
2017	**NWcap608/17**	This study	Caprine	South Africa	North West	-25.1334	26.86546	MW344008
2017	**NWbov630/17**	This study	Bovine	South Africa	North West	-26.125	23.7725	MW344009
2017	**NWbbj666/17**	This study	Black-backed jackal	South Africa	North West	-26.2802	25.10966	MW344010
2017	**GPdog574/17**	This study	Canine	South Africa	Gauteng	-25.4729	28.09919	MW344012
2017	269/17	Previously published	Canine	South Africa	North West	-26.9566	24.7284	MT454643
2017	400/17	Previously published	Canine	South Africa	North West	-26.9566	24.7284	MT454645
2017	454/17	Previously published	Black-backed jackal	South Africa	North West	-26.7167	27.1	MT454646
2017	460/17	Previously published	Black-backed jackal	South Africa	North West	-26.5961	24.17612	MT454647

2017	474/17	Previously published	Black-backed jackal	South Africa	North West	-27.5311	24.78659	MT454649
2017	477/17	Previously published	Bat-eared fox	South Africa	North West	-26.6181	25.65319	MT454650
2017	480/17	Previously published	Black-backed jackal	South Africa	North West	-27.1887	25.32931	MT454651
2017	466/17	Previously published	Black-backed jackal	South Africa	North West	-26.7167	27.1	MT454648
2017	483/17	Previously published	Black-backed jackal	South Africa	North West	-26.3138	26.89865	MT454652
2017	502/17	Previously published	Black-backed jackal	South Africa	North West	-26.7167	27.1	MT454653
2017	503/17	Previously published	Black-backed jackal	South Africa	North West	-26.7167	27.1	MT454654
2017	**LPbov354/17**	This study	Bovine	South Africa	Limpopo	-24.5917	27.41155	MW343945
2018	**NWdog44/18**	This study	Canine	South Africa	North West	-26.152	26.15968	MW343985
2018	**NWbbj110/18**	This study	Black-backed jackal	South Africa	North West	-27.1718	26.12699	MW343983
2018	**NWdog121/18**	This study	Canine	South Africa	North West	-26.4677	26.83939	MW343984
2018	**NWbbj135/18**	This study	Black-backed jackal	South Africa	North West	-26.9566	24.7284	MW343990
2018	**NWbbj195/18**	This study	Black-backed jackal	South Africa	North West	-26.2833	26.8	MW343986
2018	**NWdog270/18**	This study	Canine	South Africa	North West	-25.354	26.53009	MW344019
2018	**NWdog293/18**	This study	Canine	South Africa	North West	-25.1334	26.86546	MW344020
2018	**NWbov299/18**	This study	Bovine	South Africa	North West	-27.4377	25.13069	MW343991
2018	**NWbbj343/18**	This study	Black-backed jackal	South Africa	North West	-27.914	25.16111	MW343981
2018	**NWbov382/18**	This study	Bovine	South Africa	North West	-26.152	26.15968	MW343997
2018	**NWbbj387/18**	This study	Black-backed jackal	South Africa	North West	-26.8097	27.28492	MW343998
2018	**NWdog391/18**	This study	Canine	South Africa	North West	-26.8351	27.04304	MW343999
2018	**NWdog405/18**	This study	Canine	South Africa	North West	-25.8963	27.42684	MW344021

2018	**NWdog420/18**	This study	Canine	South Africa	North West	-25.605	27.91	MW344000
2018	**NWovi429/18**	This study	Ovine	South Africa	North West	-26.4748	27.06278	MW344001
2018	**NWjac455/18**	This study	Jackal	South Africa	North West	-26.152	26.15968	MW343978
2018	**NWgen516/18**	This study	Genet	South Africa	North West	-25.537	26.07512	MW344022
2018	**NWbef103/18**	This study	Bat-eared fox	South Africa	North West	-26.1944	24.92368	MW344013
2019	**NWbov76/19**	This study	Bovine	South Africa	North West	25.16111	24.17612	MW343969
2019	**NWbbj96/19**	This study	Black-backed jackal	South Africa	North West	-26.3138	26.89865	MW343970
2019	**NWcap103/19**	This study	Caprine	South Africa	North West	-26.8091	26.00538	MW343971
2019	**NWbov109/19**	This study	Bovine	South Africa	North West	-26.9566	24.7284	MW343972
2019	**NWbov151/19**	This study	Bovine	South Africa	North West	-25.1334	26.86546	MW343982
2019	**NWdog169/19**	This study	Canine	South Africa	North West	-26.8091	26.00538	MW343975
2019	**NWaard171/19**	This study	Aardwolf	South Africa	North West	-25.6676	27.24208	MW343976
2019	**NWdog191/19**	This study	Canine	South Africa	North West	-25.6676	27.24208	MW343973
2019	**NWbbj219/19**	This study	Black-backed jackal	South Africa	North West	-25.6676	27.24208	MW343974
2019	**NWbbj248/19**	This study	Black-backed jackal	South Africa	North West	-26.8521	26.66672	MW343977
2019	**NWjac325/19**	This study	Jackal	South Africa	North West	-25.6676	27.24208	MW343979
2019	**NWbov331/19**	This study	Bovine	South Africa	North West	-25.354	26.53009	MW343980
2019	**NWbov379/19**	This study	Bovine	South Africa	North West	-26.9333	25.41667	MW343992
2019	**NWbov380/19**	This study	Bovine	South Africa	North West	-27.2231	25.27706	MW343995
2019	**NWbov428/19**	This study	Bovine	South Africa	North West	-26.2	25.9	MW343996
2019	**GPbov309/19**	This study	Bovine	South Africa	Gauteng	-26.0858	27.77515	MW343994

Table A3: List of RABV sequences included in the phylogenetic analysis for G-L intergenic region in the NW province. Samples numbers from this study are shown in bold.

Year Sampled	Sample Number	Year Sequenced	Species	Country	Province	Latitude	Longitude	Accession number
1990	m466	Previously published	Yellow mongoose	South Africa	Free State	-27.374025	26.619959	AF079922
1990	m420	Previously published	Yellow mongoose	South Africa	North West	-27.179994	25.958625	AF079921
2003	KZNdg03.453	Previously published	Canine	South Africa	KwaZulu-Natal	-29.8579	31.0292	DQ841514
2005	19671	Previously published	African civet	Zimbabwe	-	-18.535251	32.134863	AF304188
2005	22574	Previously published	African civet	Zimbabwe	-	-18.75569	31.719546	AF304183
2012	NWdog556/12	Previously published	Canine	South Africa	North West	-25.08	27.13	MK103308
2015	KZNbov15/261	Previously published	Bovine	South Africa	KwaZulu-Natal	-29.489295	30.216652	KY681395
2016	516/16	Previously published	Canine	South Africa	North West	-25.42612	27.2243	MT454636
2016	635/16	Previously published	Canine	South Africa	North West	-25.8026	27.87506	MT454639
2017	269/17	Previously published	Canine	South Africa	North West	-26.95659	24.7284	MT454643
2017	400/17	Previously published	Canine	South Africa	North West	-26.95659	24.7284	MT454645
2017	454/17	Previously published	Black-backed jackal	South Africa	North West	-26.71667	27.1	MT454646
2017	460/17	Previously published	Black-backed jackal	South Africa	North West	-26.59613	24.17612	MT454647
2017	474/17	Previously published	Black-backed jackal	South Africa	North West	-27.53113	24.78659	MT454649

2017	477/17	Previously published	Bat-eared fox	South Africa	North West	-26.61812	25.65319	MT454650
2017	**NWbov62/17**	This study	Bovine	South Africa	North West	-26.86476	24.79046	MW343905
2017	**NWbov126/17**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343906
2017	**NWbov331/17**	This study	Bovine	South Africa	North West	-27.18871	25.32931	MW343908
2017	**NWbov604/17**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343879
2017	480/17	Previously published	Black-backed jackal	South Africa	North West	-27.18871	25.32931	MT454651
2017	**NWbov22/17**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343893
2017	**NWbov57/17**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343895
2017	**NWbov74/17**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343901
2017	**NWjac198/17**	This study	Jackal	South Africa	North West	-26.68638	25.45907	MW343907
2017	**NWcap528/17**	This study	Caprine	South Africa	North West	-27.53113	24.78659	MW343877
2017	483/17	Previously published	Black-backed jackal	South Africa	North West	-26.31379	26.89865	MT454652
2017	502/17	Previously published	Black-backed jackal	South Africa	North West	-26.71667	27.1	MT454653
2017	503/17	Previously published	Black-backed jackal	South Africa	North West	-26.71667	27.1	MT454654
2017	**NWbov59/17**	This study	Bovine	South Africa	North West	-27.18871	25.32931	MW343897
2017	**NWbov435/17**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343894
2017	466/17	Previously published	Black-backed jackal	South Africa	North West	-26.78289	27.21425	MT454648
2017	**NWovi583/17**	This study	Ovine	South Africa	North West	-27.53113	24.78659	MW343896
2017	**NWbov630/17**	This study	Bovine	South Africa	North West	-26.125	23.7725	MW343899
2017	**NWbbj666/17**	This study	Black-backed jackal	South Africa	North West	-26.28018	25.10966	MW343900
2017	**GPdog574/17**	This study	Canine	South Africa	Gauteng	-25.47288	28.09919	MW343902
2017	**NWdog17/17**	This study	Canine	South Africa	North West	-25.79053	27.2421	MW343892
2017	**NWdog31/17**	This study	Canine	South Africa	North West	-25.16092	27.16296	MW343904
2017	**NWbov432/17**	This study	Bovine	South Africa	North West	-26.17393	26.46947	MW343878
2017	**LPbov354/17**	This study	Bovine	South Africa	Limpopo	-24.59165	27.41155	MW343835

2018	**NWbef103/18**	This study	Bat-eared fox	South Africa	North West	-26.19439	24.92368	MW343861
2018	**NWdog44/18**	This study	Canine	South Africa	North West	-26.152	26.15968	MW343875
2018	**NWbbj135/18**	This study	Black-backed jackal	South Africa	North West	-26.95659	24.7284	MW343880
2018	**NWbov299/18**	This study	Bovine	South Africa	North West	-27.43774	25.13069	MW343881
2018	**NWbbj343/18**	This study	Black-backed jackal	South Africa	North West	-27.91402	25.16111	MW343871
2018	**NWbbj110/18**	This study	Black-backed jackal	South Africa	North West	-27.17179	26.12699	MW343873
2018	**NWdog121/18**	This study	Canine	South Africa	North West	-26.46765	26.83939	MW343874
2018	**NWbbj195/18**	This study	Black-backed jackal	South Africa	North West	-26.28333	26.8	MW343876
2018	**NWbbj387/18**	This study	Black-backed jackal	South Africa	North West	-26.80974	27.28492	MW343888
2018	**NWdog391/18**	This study	Canine	South Africa	North West	-26.83507	27.04304	MW343889
2018	**NWdog405/18**	This study	Canine	South Africa	North West	-25.89625	27.42684	MW343911
2018	**NWovi429/18**	This study	Ovine	South Africa	North West	-26.47483	27.06278	MW343891
2018	**NWjac455/18**	This study	Jackal	South Africa	North West	-26.152	26.15968	MW343868
2018	**NWdog270/18**	This study	Canine	South Africa	North West	-25.35397	26.53009	MW343909
2018	**NWdog293/18**	This study	Canine	South Africa	North West	-25.13342	26.86546	MW343910
2018	**NWdog420/18**	This study	Canine	South Africa	North West	-25.605	27.91	MW343890
2018	**NWgen516/18**	This study	Genet	South Africa	North West	-25.53695	26.07512	MW343912
2018	**NWbov382/18**	This study	Bovine	South Africa	North West	-26.152	26.15968	MW343887
2019	**NWbov76/19**	This study	Bovine	South Africa	North West	25.16111	24.17612	MW343859
2019	**NWbov109/19**	This study	Bovine	South Africa	North West	-26.95659	24.7284	MW343862
2019	**NWbov380/19**	This study	Bovine	South Africa	North West	-27.22308	25.27706	MW343885
2019	**NWbbj96/19**	This study	Black-backed jackal	South Africa	North West	-26.31379	26.89865	MW343860
2019	**NWcap103/19**	This study	Caprine	South Africa	North West	-26.80908	26.00538	MW343861
2019	**NWdog169/19**	This study	Canine	South Africa	North West	-26.80908	26.00538	MW343865
2019	**NWbbj248/19**	This study	Black-backed jackal	South Africa	North West	-26.85213	26.66672	MW343867
2019	**NWbov379/19**	This study	Bovine	South Africa	North West	-26.93333	25.41667	MW343882
2019	**GPbov309/19**	This study	Bovine	South Africa	Gauteng	-26.08577	27.77515	MW343884
2019	**NWbov151/19**	This study	Bovine	South Africa	North West	-25.13342	26.86546	MW343872
2019	**NWaard171/19**	This study	Aardwolf	South Africa	North West	-25.66756	27.24208	MW343866
2019	**NWdog191/19**	This study	Canine	South Africa	North West	-25.66756	27.24208	MW343863

2019	**NWbbj219/19**	This study	Black-backed jackal	South Africa	North West	-25.66756	27.24208	MW343864
2019	**NWjac325/19**	This study	Jackal	South Africa	North West	-25.66756	27.24208	MW343869
2019	**NWbov331/19**	This study	Bovine	South Africa	North West	-25.35397	26.53009	MW343870
2019	**NWbov428/19**	This study	Bovine	South Africa	North West	-26.2	25.9	MW343886

Table A4: List of RABV sequences included in the phylogenetic analysis for partial N gene in the LP province. Samples numbers from this study are shown in bold.

Year Sampled	Sample Number	Sequenced	Species	Country	Province	Latitude	Longitude	Accession number
1986	86031MOZ	Previously published	Canine	Mozambique	Unknown	Unknown	Unknown	KX148203
1988	385	Previously published	Jackal	Botswana	Ghanzi	-22	22	AY330733
1991	445	Previously published	Horse	Botswana	Maun	-19.9833	23.4167	AY330750
1991	473	Previously published	Bovine	Botswana	Serowe	-22.3875	26.7108	AY330755
1992	20639	Previously published	African civet	Zimbabwe	Macheke	-18.139	31.8493	KY553269
1994	22759	Previously published	African civet	Zimbabwe	Shamva	-17.1237	31.6415	KY553271
1997	RV1922	Previously published	Black-backed jackal	South Africa	Fraserburg	-31.9157	21.5134	DQ489878

2012	556/12	Previously published	Canine	South Africa	Mogwase	-25.2775	27.2161	KT892003
2012	433/12	Previously published	Black-backed jackal	South Africa	Polokwane	-23.9045	29.4689	KT892007
2012	LPbef227/12	Previously published	Bat-eared fox	South Africa	Limpopo	-23.667	27.8077	MW548643
2014	LPbbj536/14	Previously published	Black-backed jackal	South Africa	Limpopo	-24.884	28.3287	MW548650
2015	LPbbj391/15	Previously published	Black-backed jackal	South Africa	Limpopo	-24.351	30.9577	MW548646
2015	LPbbj475/15	Previously published	Black-backed jackal	South Africa	Limpopo	-23.92	29.4554	MW548649
2015	**LPbbj651/15**	This study	Black-backed jackal	South Africa	Limpopo	-24.9663	29.2907	MW548651
2015	682/15	Previously published	Canine	South Africa	North West	-25.354	26.5301	MT454635
2016	LPbbj237/16	Previously published	Black-backed jackal	South Africa	Limpopo	-23.943	31.1411	MW548644
2016	LPbbj264/16	Previously published	Black-backed jackal	South Africa	Limpopo	-23.743	30.1168	MW548645
2016	LPbef402/16	Previously published	Bat-eared fox	South Africa	Limpopo	-23.666	27.7448	MW548647
2017	**LPdog95/17**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343953
2017	**LPdog111/17**	This study	Canine	South Africa	Limpopo	-23.6934	30.14	MW343946
2017	**LPdog128/17**	This study	Canine	South Africa	Limpopo	-22.9456	30.485	MW343954
2017	**LPdog181/17**	This study	Canine	South Africa	Limpopo	-23.0439	29.9032	MW343947
2017	**LPdog307/17**	This study	Canine	South Africa	Limpopo	-23.4667	29.7	MW343955
2017	**LPdog318/17**	This study	Canine	South Africa	Limpopo	-23.0949	30.2908	MW343956
2017	**LPdog349/17**	This study	Canine	South Africa	Limpopo	-23.943	31.1411	MW343957
2017	**LPbov354/17**	This study	Bovine	South Africa	Limpopo	-24.5917	27.4116	MW343945
2017	**LPbov390/17**	This study	Bovine	South Africa	Limpopo	-23.2201	31.2288	MW343944
2017	**LPovi391/17**	This study	Ovine	South Africa	Limpopo	-22.6215	28.6665	MW343958
2017	**LPdog407/17**	This study	Canine	South Africa	Limpopo	-23.4093	30.1954	MW343968

2017	**LPdog422/17**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343948
2017	**LPdog425/17**	This study	Canine	South Africa	Limpopo	-23.0176	29.7984	MW343959
2017	**LPdog426/17**	This study	Canine	South Africa	Limpopo	-22.7547	30.1936	MW343960
2017	**LPdog467/17**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343961
2017	**LPdog530/17**	This study	Canine	South Africa	Limpopo	-23.2861	29.1396	MW343952
2017	**LPbov531/17**	This study	Bovine	South Africa	Limpopo	-23.3025	30.7187	MW343951
2017	**LPdog534/17**	This study	Canine	South Africa	Limpopo	-24.2848	29.8638	MW343962
2017	**LPbov564/17**	This study	Bovine	South Africa	Limpopo	-24.1667	28.6167	MW343949
2017	**LPbov592/17**	This study	Bovine	South Africa	Limpopo	-24.0685	28.0939	MW343963
2017	**LPdog603/17**	This study	Canine	South Africa	Limpopo	-23.3025	30.7187	MW343950
2017	**LPdog609/17**	This study	Canine	South Africa	Limpopo	-22.7333	31.1	MW343964
2017	**LPdog686/17**	This study	Canine	South Africa	Limpopo	-22.3488	30.0407	MW343965
2017	NWdog31/17	This study	Canine	South Africa	North West	-25.1609	27.163	MW344014
2017	NWcap608/17	This study	Caprine	South Africa	North West	-25.1334	26.8655	MW344008
2018	**LPjac96/18**	This study	Jackal	South Africa	Limpopo	-24.2051	27.9787	MW343923
2018	**LPdog197/18**	This study	Canine	South Africa	Limpopo	-23.0176	29.7984	MW343934
2018	**LPdog221/18**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343935
2018	**LPdog257/18**	This study	Canine	South Africa	Limpopo	-23.9045	29.4689	MW343936
2018	**LPdog272/18**	This study	Canine	South Africa	Limpopo	-23.0176	29.7984	MW343937
2018	**LPbov273/18**	This study	Bovine	South Africa	Limpopo	-23.0176	29.7984	MW343938
2018	**LPdog292/18**	This study	Canine	South Africa	Limpopo	-22.7457	30.5093	MW343939
2018	**LPdog365/18**	This study	Canine	South Africa	Limpopo	-23.3025	30.7187	MW343931
2018	**LPdog370/18**	This study	Canine	South Africa	Limpopo	-23.6661	27.7448	MW343918
2018	**LPbov406/18**	This study	Bovine	South Africa	Limpopo	-23.5703	28.4341	MW343919
2018	**LPjac411/18**	This study	Jackal	South Africa	Limpopo	-23.9045	29.4689	MW343920
2018	**LPdog417/18**	This study	Canine	South Africa	Limpopo	-23.498	29.5672	MW343940
2018	**LPdog428/18**	This study	Canine	South Africa	Limpopo	-23.288	29.1368	MW343921
2018	**LPjac461/18**	This study	Jackal	South Africa	Limpopo	-24.3474	29.0388	MW343922
2018	**LPdog485/18**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343927
2018	**LPdog507/18**	This study	Canine	South Africa	Limpopo	-22.75	30.2167	MW343967

2018	**LPdog531/18**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343941
2018	**LPdog555/18**	This study	Canine	South Africa	Limpopo	-23.9833	30.2	MW343942
2018	**LPdog560/18**	This study	Canine	South Africa	Limpopo	-23.0176	29.7984	MW343943
2018	NWdog293/18	This study	Canine	South Africa	North West	-25.1334	26.8655	MW344020
2018	NWdog420/18	This study	Canine	South Africa	North West	-25.605	27.91	MW344000
2019	**LPdog65/19**	This study	Canine	South Africa	Limpopo	-23.9045	29.4689	MW343913
2019	**LPdog113/19**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343915
2019	**LPdog138/19**	This study	Canine	South Africa	Limpopo	-23.0439	29.9032	MW343924
2019	**LPdog197/19**	This study	Canine	South Africa	Limpopo	-23.0439	29.9032	MW343916
2019	**LPdog228/19**	This study	Canine	South Africa	Limpopo	-22.5587	30.828	MW343929
2019	**LPdog245/19**	This study	Canine	South Africa	Limpopo	-23.0439	29.9032	MW343917
2019	**LPdog246/19**	This study	Canine	South Africa	Limpopo	-23.0439	29.9032	MW343928
2019	**LPdog267/19**	This study	Canine	South Africa	Limpopo	-22.5587	30.828	MW343925
2019	**LPdog290/19**	This study	Canine	South Africa	Limpopo	-23.6934	30.14	MW343926
2019	**LPdog307/19**	This study	Canine	South Africa	Limpopo			MW343930
2019	**LPdog314/19**	This study	Canine	South Africa	Limpopo	-23.8332	30.1635	MW343966
2019	**LPbov329/19**	This study	Bovine	South Africa	Limpopo	-24.1944	29.0097	MW343933
2019	**LPdog335/19**	This study	Canine	South Africa	Limpopo	-23.0439	29.9032	MW343932
2019	NWaard171/19	This study	Aardwolf	South Africa	North West	-25.6676	27.2421	MW343976
2019	NWbbj219/19	This study	Black-backed jackal	South Africa	North West	-25.6676	27.2421	MW343974
2019	NWbov151/19	This study	Bovine	South Africa	North West	-25.1334	26.8655	MW343982
2019	NWdog191/19	This study	Canine	South Africa	North West	-25.6676	27.2421	MW343973
2019	NWjac325/19	This study	Jackal	South Africa	North West	-25.6676	27.2421	MW343979
2009	RV2503	Previously published	Wild Cat	Tanzania	Serengeti	-2.03961	33.714	KR906740
2011	RV2862	Previously published	Canine	Tanzania	Serengeti	-2.03961	33.714	KR906768
2011	RV2907	Previously published	Bovine	Tanzania	Serengeti	-2.03961	33.714	KR906776

Table A5: List of RABV sequences included in the phylogenetic analysis for G-L intergenic region in the LP province. Samples numbers from this study are shown in bold

Year Sampled	Sample Number	Sequenced	Species	Country	Province	Latitude	Longitude	Accession number
1986	86031MOZ	Previously published	Canine	Mozambique	Unknown	Unknown	Unknown	KX148203
2009	RV2503	Previously published	Wild Cat	Tanzania	Serengeti	-2.03961	33.71395	KR906740
2011	RV2862	Previously published	Canine	Tanzania	Serengeti	-2.03961	33.71395	KR906768
2011	RV2907	Previously published	Bovine	Tanzania	Serengeti	-2.03961	33.71395	KR906776
2012	LPbef227/12	Previously published	Bat-eared fox	South Africa	Limpopo	-23.6667	27.8077	MK098244
2012	LPbbj433/12	Previously published	Black-backed jackal	South Africa	Limpopo	-24	29	MK098232
2012	NWdog556/12	Previously published	Canine	South Africa	North West	-25.08	27.13	MK103308

2014	LPbbj536/14	Previously published	Black-backed jackal	South Africa	Limpopo	-24.884447	28.3287106	MK098254
2015	682/15	Previously published	Canine	South Africa	North West	-25.35397	26.53009	MT454635
2015	LPbbj391/15	Previously published	Black-backed jackal	South Africa	Limpopo	-24.350604	30.9576681	MK098238
2015	LPbbj475/15	Previously published	Black-backed jackal	South Africa	Limpopo	-23.92	29.4554	MK098240
2015	**LPbbj651/15**	This study	Black-backed jackal	South Africa	Limpopo	-24.31043	30.81326	MW548654
2016	LPbef402/16	Previously published	Bat-eared fox	South Africa	Limpopo	-23.666466	27.7448285	MK098253
2016	LPbbj237/16	Previously published	Black-backed jackal	South Africa	Limpopo	-23.943	31.1411	MK098251
2016	LPbbj264/16	Previously published	Black-backed jackal	South Africa	Limpopo	-23.7432	30.11681	MK098236
2017	**LPdog95/17**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343843
2017	**LPdog111/17**	This study	Canine	South Africa	Limpopo	-23.69339	30.14002	MW343836
2017	**LPdog128/17**	This study	Canine	South Africa	Limpopo	-22.94564	30.48497	MW343844
2017	**LPdog181/17**	This study	Canine	South Africa	Limpopo	-23.04385	29.90319	MW343837
2017	**LPdog307/17**	This study	Canine	South Africa	Limpopo	-23.46667	29.7	MW343845
2017	**LPdog318/17**	This study	Canine	South Africa	Limpopo	-23.09489	30.2908	MW343846
2017	**LPdog349/17**	This study	Canine	South Africa	Limpopo	-23.94299	31.14107	MW343847
2017	**LPbov354/17**	This study	Bovine	South Africa	Limpopo	-24.59165	27.41155	MW343835
2017	**LPbov390/17**	This study	Bovine	South Africa	Limpopo	-23.22013	31.22875	MW343834
2017	**LPovi391/17**	This study	Ovine	South Africa	Limpopo	-22.6215	28.66646	MW343848
2017	**LPdog407/17**	This study	Canine	South Africa	Limpopo	-23.40927	30.19538	MW343858
2017	**LPdog422/17**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343838
2017	**LPdog425/17**	This study	Canine	South Africa	Limpopo	-23.01759	29.79838	MW343849
2017	**LPdog426/17**	This study	Canine	South Africa	Limpopo	-22.75467	30.1936	MW343850
2017	**LPdog467/17**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343851
2017	**LPdog530/17**	This study	Canine	South Africa	Limpopo	-23.28609	29.13964	MW343842
2017	**LPbov531/17**	This study	Bovine	South Africa	Limpopo	-23.30246	30.71868	MW343841

2017	**LPdog534/17**	This study	Canine	South Africa	Limpopo	-24.28477	29.86381	MW343852
2017	**LPbov564/17**	This study	Bovine	South Africa	Limpopo	-24.16667	28.61667	MW343839
2017	**LPbov592/17**	This study	Bovine	South Africa	Limpopo	-24.06847	28.0939	MW343853
2017	**LPdog603/17**	This study	Canine	South Africa	Limpopo	-23.30246	30.71868	MW343840
2017	**LPdog609/17**	This study	Canine	South Africa	Limpopo	-22.73333	31.1	MW343854
2017	**LPdog686/17**	This study	Canine	South Africa	Limpopo	-22.34881	30.04074	MW343855
2017	NWdog31/17	This study	Canine	South Africa	North West	-25.16092	27.16296	MW343904
2017	NWcap608/17	This study	Caprine	South Africa	North West	-25.13342	26.86546	MW343898
2018	**LPjac96/18**	This study	Jackal	South Africa	Limpopo	-24.20514	27.9787	MW343813
2018	**LPdog197/18**	This study	Canine	South Africa	Limpopo	-23.01759	29.79838	MW343824
2018	**LPdog221/18**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343825
2018	**LPdog257/18**	This study	Canine	South Africa	Limpopo	-23.90449	29.46885	MW343826
2018	**LPdog272/18**	This study	Canine	South Africa	Limpopo	-23.01759	29.79838	MW343827
2018	**LPbov273/18**	This study	Bovine	South Africa	Limpopo	-23.01759	29.79838	MW343828
2018	**LPdog292/18**	This study	Canine	South Africa	Limpopo	-22.74567	30.50933	MW343829
2018	**LPdog365/18**	This study	Canine	South Africa	Limpopo	-23.30246	30.71868	MW343821
2018	**LPdog370/18**	This study	Canine	South Africa	Limpopo	-23.66607	27.74477	MW343808
2018	**LPbov406/18**	This study	Bovine	South Africa	Limpopo	-23.57034	28.43408	MW343809
2018	**LPjac411/18**	This study	Jackal	South Africa	Limpopo	-23.90449	29.46885	MW343810
2018	**LPdog417/18**	This study	Canine	South Africa	Limpopo	-23.49795	29.56722	MW343830
2018	**LPdog428/18**	This study	Canine	South Africa	Limpopo	-23.28799	29.13683	MW343811
2018	**LPjac461/18**	This study	Jackal	South Africa	Limpopo	-24.34735	29.03883	MW343812
2018	**LPdog485/18**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343817
2018	**LPdog507/18**	This study	Canine	South Africa	Limpopo	-22.75	30.21667	MW343857
2018	**LPdog531/18**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343831
2018	**LPdog555/18**	This study	Canine	South Africa	Limpopo	-23.98333	30.2	MW343832
2018	**LPdog560/18**	This study	Canine	South Africa	Limpopo	-23.01759	29.79838	MW343833
2018	NWdog420/18	This study	Canine	South Africa	North West	-25.605	27.91	MW343890
2018	NWdog293/18	This study	Canine	South Africa	North West	-25.13342	26.86546	MW343910

2019	**LPdog65/19**	This study	Canine	South Africa	Limpopo	-23.90449	29.46885	MW343803
2019	**LPdog113/19**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343805
2019	**LPdog138/19**	This study	Canine	South Africa	Limpopo	-23.04385	29.90319	MW343814
2019	**LPdog197/19**	This study	Canine	South Africa	Limpopo	-23.04385	29.90319	MW343806
2019	**LPdog228/19**	This study	Canine	South Africa	Limpopo	-22.55868	30.82795	MW343819
2019	**LPdog245/19**	This study	Canine	South Africa	Limpopo	-23.04385	29.90319	MW343807
2019	**LPdog246/19**	This study	Canine	South Africa	Limpopo	-23.04385	29.90319	MW343818
2019	**LPdog267/19**	This study	Canine	South Africa	Limpopo	-22.55868	30.82795	MW343815
2019	**LPdog290/19**	This study	Canine	South Africa	Limpopo	-23.69339	30.14002	MW343816
2019	**LPdog307/19**	This study	Canine	South Africa	Limpopo	Unknown	Unknown	MW343820
2019	**LPdog314/19**	This study	Canine	South Africa	Limpopo	-23.83322	30.16351	MW343856
2019	**LPbov329/19**	This study	Bovine	South Africa	Limpopo	-24.19436	29.00974	MW343823
2019	**LPdog335/19**	This study	Canine	South Africa	Limpopo	-23.04385	29.90319	MW343822
2019	NWbov151/19	This study	Bovine	South Africa	North West	-25.13342	26.86546	MW343872
2019	NWaard171/19	This study	Aardwolf	South Africa	North West	-25.66756	27.24208	MW343866
2019	NWdog191/19	This study	Canine	South Africa	North West	-25.66756	27.24208	MW343863
2019	NWbbj219/19	This study	Black-backed jackal	South Africa	North West	-25.66756	27.24208	MW343864
2019	NWjac325/19	This study	Jackal	South Africa	North West	-25.66756	27.24208	MW343869

Figures

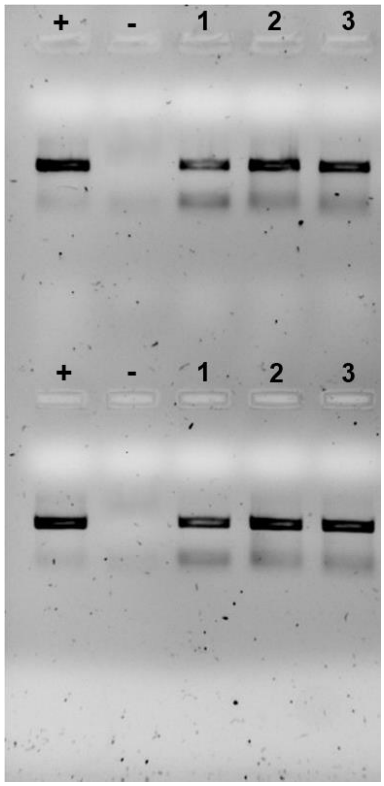

Figure A1: Gel electrophoresis of samples used for the Taguchi optimisation of the partial N gene PCR reaction. The top part of the gel shows the results from reaction 7 in the Taguchi table, while the bottom part shows the results from a hybrid protocol using the parameters from both reaction 7 and 9. +: positive control, -: negative control, 1, 2, and 3: samples 1 – 3 used for each reaction