

# Evidence for a host switching in the maintenance of canid rabies variant in two wild carnivore species in the Northern Cape Province, South Africa

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Rabies is a zoonotic infectious disease that causes at least 59 000 human deaths worldwide annually, with 95% of the cases occurring in the developing countries of Asia and Africa. There are two *Lyssavirus rabies* (RABV) variants circulating in South Africa, notably the canid and mongoose RABV biotypes. The canid RABV biotype is maintained in the domestic dog and two wild carnivore species, the black-backed jackal (*Canis mesomelas*) and the bat-eared fox (*Otocyon megalotis*). The yellow mongoose, a member of the *Herpestidae* family, is a reservoir and vector species for the mongoose RABV biotype. Rabies trends showed an increase in rabies-positive cases in aardwolves between 2011 and 2016 surpassing the bat-eared fox as the most rabies-affected wild carnivore in the Northern Cape Province of South Africa. The aim of the study was to establish the genetic relationships amongst rabies viruses recovered from both the aardwolves and bat-eared foxes. A partial region of the glycoprotein gene and the variable G-L intergenic region of the viral genome were analysed using nucleotide sequences generated from PCR amplicons. The rabies viruses recovered from the aardwolves between the year 2015 and 2017 were 100% nucleotide sequence identical, suggesting a single or common source and possible evidence for a host shift. Furthermore, the phylogenetic reconstruction demonstrated that the rabies viruses obtained from the two wild carnivore species from the Northern Cape Province clustered independently of each other with 96% nucleotide sequence identity, suggesting that the aardwolf may be able to maintain the canid RABV variant in this geographical area.

**Keywords:** rabies, wildlife, aardwolf, phylogenetic analysis, Northern Cape Province

## Introduction

Rabies is a neglected zoonotic and fatal disease of warm-blooded vertebrates including humans. Death is inevitable as soon as clinical signs develop. Rabies remains a significant public and veterinary health problem causing at least 59 000 human deaths annually and primarily affecting developing countries in Asia and Africa (Hampson et al. 2015). The causative agent of the disease is a viral species of the *Lyssavirus* genus currently composed of 17 confirmed viral species (Walker et al. 2020). Recently, putative and unclassified lyssaviruses named Kotalahti bat lyssavirus (KBLV) that were isolated from a Brandt's bat (*Myotis brandtii*) in Finland (Nokireki et al. 2018), and Matlo bat lyssavirus isolated from Natal long-fingered bat (*Miniopterus natalensis*) in South Africa (Coertse et al. 2020), both await formal inclusion into the genus. At least four members of the *Lyssavirus* genus have been identified and confirmed in terrestrial and Chiropteran host species in South Africa, including the classical *Lyssavirus rabies* (RABV), *Lyssavirus Lagos* (LBV), *Lyssavirus mokola* (MOKV) and *Lyssavirus duvenhage* (DUVV) (Walker et al. 2022).

Epidemiologically, RABV is distributed globally with the exception of some nation islands (such as Solomon Islands, Marshall Islands, Papua New Guinea, etc. (World Health Organization 2022)). This highly neurotropic pathogen has a wide host range including terrestrial mammals and several bat species (Badrane & Tordo 2001). In southern Africa, RABV occurs as two distinct variants adapted to *Carnivora* and *Herpestidae* families, and

these are referred to as the canid and mongoose RABV biotypes respectively (King et al. 1993; von Teichman et al. 1995). The canid RABV biotype is highly adapted and transmitted primarily by domestic dogs and is also maintained by wild carnivore species such as the black-backed jackal (*Canis mesomelas*) and bat-eared fox (*Otocyon megalotis*), whereas the mongoose RABV biotype is transmitted by members of the *Herpestidae* family, especially the yellow mongoose (*Cynictis penicillata*). It has been shown that black-backed jackals are able to maintain and transmit RABV in the north-western regions of South Africa (Zulu et al. 2007) where ecological conditions are favourable and support the growth of the vector populations. On the other hand, bat-eared foxes maintain and transmit the RABV in the western and drier regions of the country, including the Northern and Western Cape provinces respectively (Sabeta et al. 2007).

The aardwolf (*Proteles cristatus*) is a member of the *Hyaenidae* family that weighs 8–12 kg, and thrives on termites (Skinner & Chimimba 2005). This wild carnivore species is generally found in the semi-arid areas of southern Africa (Koehler & Richardson 1990) and its distribution is influenced primarily by the availability of termites of the genus *Trinervitermes*, which constitute its main diet (Cooper & Skinner 1979; Richardson 1987). Aardwolves escape the thermal stresses of the environment they occupy by making extensive use of underground dens during the inactive periods of the colder months (Anderson 1994; Richardson 1987). It is believed that during the summer and winter months, aardwolves spend more than half a day in underground dens

(Anderson 2004). Furthermore, during the colder months, aardwolves switch to diurnal feeding on a different termite species, *Hodotermes mossambicus* (Richardson 1987), which is the primary food source for the bat-eared fox (Sillero-Zuberi 2009). Dens are also important for the aardwolves especially during the rearing of the young cubs and act as a refuge for the young cubs from predators such as black-backed jackals (Richardson 1985). Aardwolves frequently relocate to new dens when they have young cubs (every 2–5 days), a behaviour believed to be an anti-predatory strategy for protecting their young cubs (Richardson 1985).

Historically, rabies in the Northern Cape Province has been mainly confirmed in the bat-eared fox and members of the *Herpestidae* family, especially the yellow mongoose (ARC-OVR, unpublished records). The sporadic cases of rabies were recorded in bat-eared foxes from 1955, and since 1980 onwards there have been up to 24 confirmed cases each year (Swanepoel et al. 1993). The epidemiological and surveillance data showed a steady increase in the number of rabies-positive cases reported in aardwolves in the Northern Cape Province, South Africa. Furthermore, surveillance data showed that the number of positive rabies cases reported in aardwolves has surpassed those obtained from the bat-eared fox from 2011 (ARC-OVR, unpublished records). The rabies virus is a multi-host pathogen, to which all mammals are susceptible. Cross-species transmission or spill-over events occur when the rabies virus, which has adapted to a specific animal reservoir species is transmitted to a non-reservoir species (Wallace et al. 2014). Most cross-species transmission events do not lead to successful onward transmissions or infections within a non-reservoir population and are referred to as dead-end infections (Guerra et al. 2003; Kim et al. 2013). The rabies virus maintenance cycle in a particular host species represents a cross-species transmission event that occurred in the past and has established transmission in a novel host species (Mollentze et al. 2020). Elsewhere, it has been shown that repeated cross-species transmission from bats to skunks (*Mephitis mephitis*) and grey foxes (*Urocyon cinereoargenteus*) in northern Arizona resulted in a focal region of sustained bat rabies variant transmission within these terrestrial mammals (Kuzmin et al. 2012; Wallace et al. 2014).

The cytoplasmic domain of glycoprotein gene and G-L intergenic region is the most genetically divergent portion of the RABV genome (Sacramento et al. 1991) and its relevance in monitoring epidemiological changes in the evolution of RABVs has been investigated and routinely used globally, including in South Africa (Von Teichman et al. 1995; Ngoepe et al. 2009). Our primary objective was to establish the genetic relationships of the RABV isolates originating from aardwolves with those obtained from bat-eared foxes. Secondly, to establish the origin of the rabies epizootic in the aardwolf population. In this way, we attempted to clarify whether rabies in aardwolves is indeed part of a new and independent rabies epidemiological cycle.

## Materials and methods

### Viruses

A cohort of 52 RABV isolates collected from aardwolves between 1994 and 2017 were selected from the rabies repository at the Agricultural Research Council, Onderstepoort Veterinary Research (ARC-OVR) (Pretoria, South Africa) (Table I) for this study. The state veterinarians submit rabies-suspected cases to the laboratory for confirmation as part of the national and rabies passive surveillance programme in South Africa. The specimens were initially shown to contain lyssavirus antigen by the direct fluorescent antibody test (dFAT) (Rupprecht et al. 2018) prior to genetic characterisation. The rabies viruses collected between 1994 and 1998 were passaged once in suckling mice and subsequently stored as 20% lyophilised mouse-infected brain tissues. Viruses collected after 1998 were stored as original brain tissues at -70 °C. An additional nine rabies viruses obtained from bat-eared foxes collected between 2016 and 2017 were included in the study as well as previously published nucleotide sequences of rabies viruses obtained from the Northern and Western Cape Provinces of South Africa (Table I). Microsoft Excel version 2019 (Microsoft Corporation, 2018) was used for data management and descriptive statistics.

### Viral RNA extraction, RT-PCR and sequencing

Total viral RNA was extracted from either the original infected brain tissues or 20% lyophilised mouse brain tissues using Tri-Reagent (Sigma Aldrich, USA) followed by Directzol™ RNA miniprep kit (Zymo Research, USA) according to the manufacturer's instructions. The extracted RNA was stored at -80 °C until required. A reverse transcription polymerase chain reaction (RT-PCR) was performed using the G (+) and L (-) primer set targeting an 860 bp region inclusive of the cytoplasmic domain of the glycoprotein and the G-L intergenic region of the viral genome (Sacramento et al. 1991). The PCR products were electrophoresed in 1% agarose gels and subsequently purified using the PCR purification kit (Qiagen, Germany) according to the manufacturer's protocol. The purified PCR amplicons were sequenced in both directions using the G (+) and L (-) primers as in the PCR reactions with the BigDye<sup>®</sup> Terminator v3.1 sequencing reaction kit (Applied Biosystems, USA) on an ABI 3100 automated sequencer (Applied Biosystems, USA).

### Phylogenetic analysis

The phylogenetic analysis included the nucleotide sequences described in this study and other previously characterised rabies virus sequences in bat-eared foxes originating from the Northern Cape Province and the neighbouring Western Cape Province (Table I). Nucleotide sequences ( $n = 87$ ) from both the Northern Cape and Western Cape provinces were aligned using ClustalW subroutine of MEGA X software package (Kumar et al. 2018) and the best fitting nucleotide substitution model was found to be symmetrical model plus Gamma (SYM + G) using the Akaike's information criterion (AIC) subroutine of the j-Model test software package (version 2.1.10). The phylogenetic analysis was undertaken using a Bayesian Markov Chain Monte Carlo (MCMC) method in the BEAST software package (version 2.5.0) using a relaxed exponential clock (Bouckaert et al. 2019).

**Table 1:** The epidemiological information of *Lyssavirus rabies* isolates obtained from bat-eared foxes (*Otocyon megalotis*) and aardwolves (*Proteles cristatus*) in the Northern Cape and Western Cape Provinces included in the study

Virus number	Lab number	Year obtained	Nearest town	Species	Locality	Accession number	Reference
1	08/94	1994	De Aar	<i>Proteles cristatus</i>	De Aar	MK467461	This study
2	477/94	1994	Van Rhynsdorp	<i>Proteles cristatus</i>	Van Rhynsdorp	MK467493	This study
3	835/94	1994	Carnarvon	<i>Proteles cristatus</i>	Wolwerf	MK467463	This study
4	383/96	1996	Albany	<i>Proteles cristatus</i>	Palmetfontein	MK467464	This study
5	163/97	1997	Hopetown	<i>Proteles cristatus</i>	Pienaarspan	MK467465	This study
6	414/98	1998	Calvinia	<i>Proteles cristatus</i>	Kaapsvlei farm	MK467466	This study
7	296/00	2000	Kenhardt	<i>Proteles cristatus</i>	Brulpan	MK467467	This study
8	302/00	2000	Hay	<i>Proteles cristatus</i>	Kameeldam	MK467468	This study
9	620/00	2000	Kenhardt	<i>Proteles cristatus</i>	Oubip	MK467469	This study
10	643/00	2000	Springbok	<i>Proteles cristatus</i>	Kamasoas	MK467470	This study
11	249/01	2001	Britstown	<i>Proteles cristatus</i>	Twyfelhoek	MK467471	This study
12	033/04	2004	Springbok	<i>Proteles cristatus</i>	Kamasoas	MK467472	This study
13	064/06	2006	Upington	<i>Proteles cristatus</i>	Karamas	MK467473	This study
14	146/06	2006	Hope	<i>Proteles cristatus</i>	Gannahoek	MK467474	This study
15	407/06	2006	Springbok	<i>Proteles cristatus</i>	Koamsvlei	MK467475	This study
16	534/06	2006	Britstown	<i>Proteles cristatus</i>	Nuwejaarsfontein	MK467476	This study
17	578/06	2006	Springbok	<i>Proteles cristatus</i>	Aggeneys	MK467477	This study
18	228/07	2007	Springbok	<i>Proteles cristatus</i>	Namaqua	MK467478	This study
19	404/07	2007	Gordonia	<i>Proteles cristatus</i>	De Oudeputs	MK467479	This study
20	430/09	2009	Springbok	<i>Proteles cristatus</i>	Van Tiddensville	MK467480	This study
21	718/09	2009	Hanover	<i>Proteles cristatus</i>	Beestekuil	MK467481	This study
22	848/09	2009	Britstown	<i>Proteles cristatus</i>	Westfront	MK467482	This study
23	774/11	2011	Springbok	<i>Proteles cristatus</i>	Soutvlei	MK467483	This study
24	906/11	2011	Springbok	<i>Proteles cristatus</i>	Dikbek	MK467484	This study
25	60/13	2013	Springbok	<i>Proteles cristatus</i>	Springbok	MK467485	This study
26	264/13	2013	Springbok	<i>Proteles cristatus</i>	Driegat	MK467486	This study
27	320/13	2013	Springbok	<i>Proteles cristatus</i>	Rooiduin	MK467487	This study
28	55/14	2014	Springbok	<i>Proteles cristatus</i>	Gamoep	MK467488	This study
29	525/14	2014	Springbok	<i>Proteles cristatus</i>	Skietpoort	MK467489	This study
30	619/14	2014	Upington	<i>Proteles cristatus</i>	Swartoup	MK467490	This study
31	737/14	2014	Carnarvon	<i>Proteles cristatus</i>	Vredelus	MK467491	This study
32	155/15	2015	Springbok	<i>Proteles cristatus</i>	Soutvlei	MK467492	This study
33	315/15	2015	Springbok	<i>Proteles cristatus</i>	Heuningvlei	MK467493	This study
34	360/15	2015	Upington	<i>Proteles cristatus</i>	Spitskop	MK467494	This study
35	361/15	2015	Upington	<i>Proteles cristatus</i>	De Bome	MK467495	This study
36	372/15	2015	Gordonia	<i>Proteles cristatus</i>	Abeam	MK467496	This study
37	551/15	2015	Springbok	<i>Proteles cristatus</i>	Aggeneys	MK467497	This study
38	802/15	2015	Marydale	<i>Proteles cristatus</i>	Brakbospoort	MK467498	This study
39	124/16	2016	De Aar	<i>Proteles cristatus</i>	Nuwejaarsfontein	MK467499	This study
40	145/16	2016	Gordonia	<i>Proteles cristatus</i>	Upington	MK467500	This study
41	174/16	2016	Springbok	<i>Proteles cristatus</i>	Kamasoas	MK467501	This study
42	212/16	2016	Springbok	<i>Proteles cristatus</i>	Kamasoas	MK467502	This study
43	345/16	2016	De Aar	<i>Proteles cristatus</i>	Perdepoort	MK467503	This study
44	362/16	2016	Springbok	<i>Proteles cristatus</i>	Kamasoas	MK467504	This study
45	478/16	2016	Springbok	<i>Proteles cristatus</i>	Kamasoas	MK467505	This study
46	526/16	2016	Springbok	<i>Proteles cristatus</i>	Aggeneys	MK467506	This study
47	570/16	2016	Springbok	<i>Proteles cristatus</i>	Dikbek	MK467507	This study
48	725/16	2016	Springbok	<i>Proteles cristatus</i>	Rooiduin	MK467508	This study

49	805/16	2016	Springbok	<i>Proteles cristatus</i>	Aggeneys	MK467509	This study
50	305/17	2017	Springbok	<i>Proteles cristatus</i>	Taaibosmond	MK467510	This study
51	389/17	2017	De Aar	<i>Proteles cristatus</i>	Loskop	MK467511	This study
52	399/17	2017	Britstown	<i>Proteles cristatus</i>	Westfront	MK467512	This study
53	425/16	2016	Springbok	<i>Otocyon megalotis</i>	Oubib	MK467513	This study
54	630/16	2016	Upington	<i>Otocyon megalotis</i>	Bloubos	MK467514	This study
55	631/16	2016	Upington	<i>Otocyon megalotis</i>	Bloubos	MK467515	This study
56	676/16	2016	Calvinia	<i>Otocyon megalotis</i>	Uitkyk	MK467516	This study
57	768/16	2016	De Aar	<i>Otocyon megalotis</i>	Rietpoort	MK467517	This study
58	899/16	2016	De Aar	<i>Otocyon megalotis</i>	Lubberhoek	MK467518	This study
59	273/17	2017	Upington	<i>Otocyon megalotis</i>	Kalahari Monate Lodge	MK467519	This study
60	368/17	2017	Kuruman	<i>Otocyon megalotis</i>	Lucas Dam	MK467520	This study
61	374/17	2017	Calvinia	<i>Otocyon megalotis</i>	Bo Downes	MK467521	This study
62	1546/80	1980	Gordonia	<i>Otocyon megalotis</i>	Not available	DQ431357	Sabeta et al. 2007
63	460/94	1994	Namaqualand	<i>Otocyon megalotis</i>	Black mountain mine	DQ431397	Sabeta et al., 2007
64	750/94	1994	Namaqualand	<i>Otocyon megalotis</i>	Koppieskraal	DQ431386	Sabeta et al., 2007
65	278/99	1999	Britstown	<i>Otocyon megalotis</i>	Not available	DQ431281	Sabeta et al. 2007
66	201/01	2001	De Aar	<i>Otocyon megalotis</i>	Vrede farm	DQ431279	Sabeta et al. 2007
67	271/04	2004	Britstown	<i>Otocyon megalotis</i>	Rietpoort	DQ431275	Sabeta et al. 2007
68	681/00	2000	Gordonia	<i>Otocyon megalotis</i>	Mata Mata farm	DQ431353	Sabeta et al. 2007
69	312/03	2003	De Aar	<i>Otocyon megalotis</i>	Grootfourieskolk	DQ431381	Sabeta et al. 2007
70	385/01	2001	Malmesbury	<i>Otocyon megalotis</i>	Kleygat farm	DQ431381	Sabeta et al. 2007
71	95/02	2002	Malmesbury	<i>Otocyon megalotis</i>	Ronde bossie farm	DQ431382	Sabeta et al. 2007
72	05/71	2005	Vredendal	<i>Otocyon megalotis</i>	Not available	DQ431385	Sabeta et al. 2007
73	04/137	2004	Moorresburg	<i>Otocyon megalotis</i>	Not available	DQ431369	Sabeta et al. 2007
74	04/157	2004	Piketberg	<i>Otocyon megalotis</i>	Not available	DQ431367	Sabeta et al. 2007
75	04/72	2004	Piketberg	<i>Otocyon megalotis</i>	Not available	DQ431359	Sabeta et al. 2007
76	548/00	2000	Clanwilliam	<i>Otocyon megalotis</i>	Bergsoom farm	DQ431368	Sabeta et al. 2007
77	236/00	2000	Malmesbury	<i>Otocyon megalotis</i>	Jonaskloof farm	DQ431375	Sabeta et al. 2007
78	449/94	1994	Malmesbury	<i>Otocyon megalotis</i>	Klipfontein	DQ431374	Sabeta et al. 2007
79	04/424	2004	Malmesbury	<i>Otocyon megalotis</i>	Not available	DQ431377	Sabeta et al. 2007
80	04/489	2004	Moorresburg	<i>Otocyon megalotis</i>	Not available	DQ431378	Sabeta et al. 2007
81	53/00	2000	Malmesbury	<i>Otocyon megalotis</i>	Goedehoop farm	DQ431371	Sabeta et al. 2007
82	266/12	2012	Herbert	<i>Otocyon megalotis</i>	Herbert	MK098226	Schepers, C. 2018
83	313/12	2012	De Aar	<i>Otocyon megalotis</i>	Driecoppen	MK098225	Schepers, C. 2018
84	149/14	2014	Calvinia	<i>Otocyon megalotis</i>	Ramskop	MK098229	Schepers, C. 2018
85	444/15	2015	Kuruman	<i>Otocyon megalotis</i>	Gannavlake	MK098227	Schepers, C. 2018
86	554/15	2015	Kuruman	<i>Otocyon megalotis</i>	Gannavlake	MK098222	Schepers, C. 2018

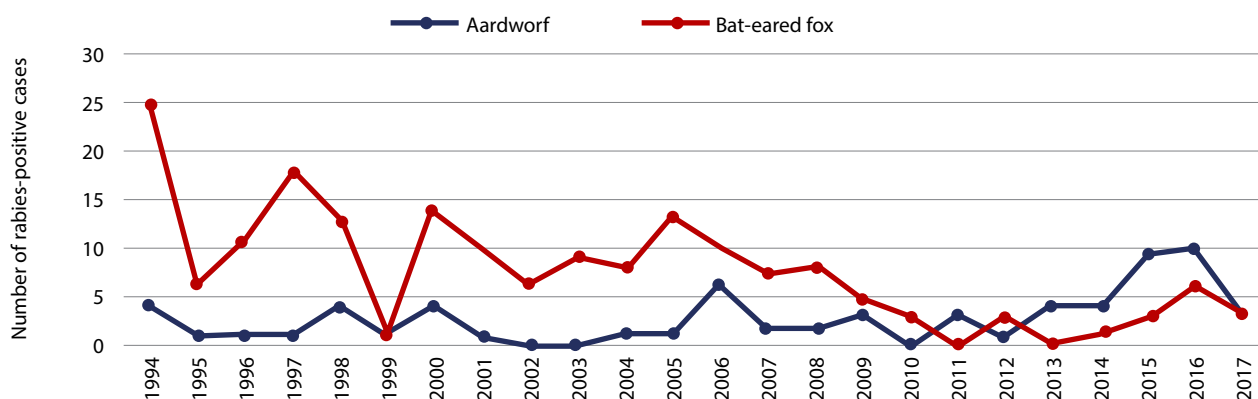
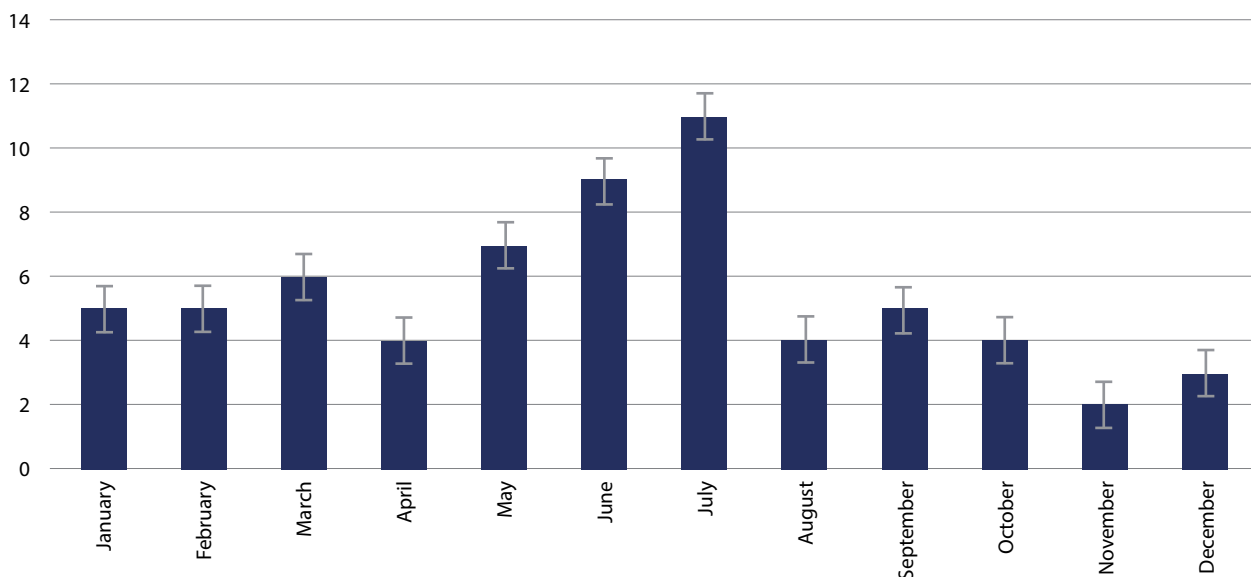


Figure 1: Comparison of rabies-positive cases confirmed in the aardworf and bat-eared fox from the Northern Cape Province between the year 1994 and 2017



**Figure 2:** Average monthly rabies cases confirmed in the aardwolf in the Northern Cape Province, South Africa (1994–2017)

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 10% of burn-in. The posterior distributions were subsequently inspected using the Tracer software (version 1.7.1) and the results were summarised as a maximum clade credibility tree and visualised using FigTree software (version 1.4.4). In addition, the pairwise mean evolutionary distances were calculated using Kimura-2 parameter distance model with a bootstrap of 1 000 replicates subroutine of MEGA X software package (Nei & Kumar 2000).

## Results

### *Rabies statistics and trends in selected study species from 1994–2017*

The epidemiological and historical surveillance data at the World Organization for Animal Health (WOAH) Rabies Reference Laboratory showed a steady increase in rabies-positive cases reported in aardwolves from 2011 until 2017 from the Northern Cape Province in South Africa (Figure 1). The rabies-positive cases diagnosed in bat-eared foxes and aardwolves between 1994 and 2017 represented 36% ( $n = 195$ ) and 12% ( $n = 67$ ) positivity rates respectively (Figure 1). In addition, the trends analyses demonstrated that the rabies-positive cases in bat-eared foxes were six times higher than the rabies-positive cases recorded in aardwolves between 1994 and 2010 (Figure 1). Additionally, the data demonstrated that from 2011 until 2016 aardwolves were the most affected wildlife carnivore species infected with the rabies virus as compared to bat-eared foxes (Figure 1). In addition, the passive surveillance data revealed that rabies-positive cases in aardwolves increased during the dry winter season, peaking in July on average (Figure 2).

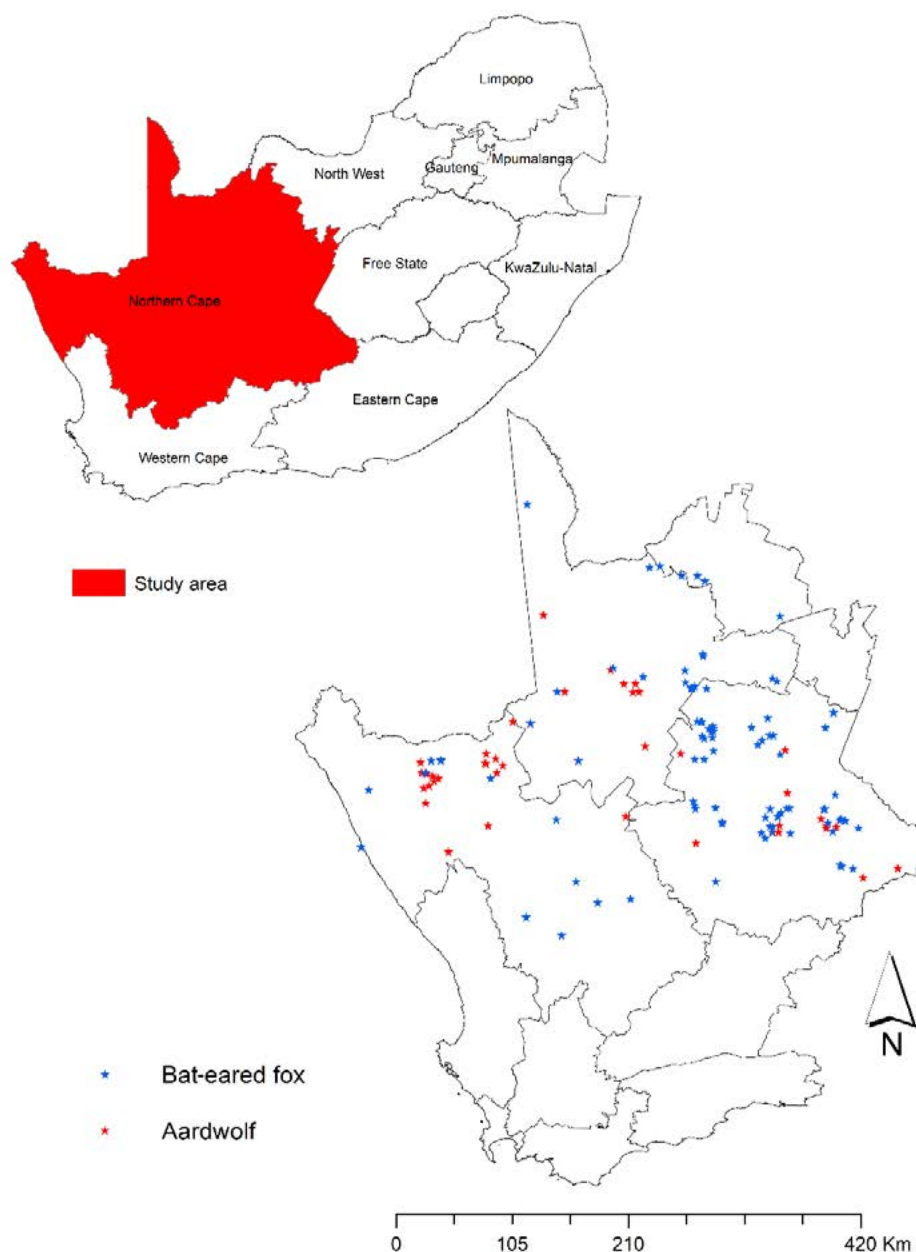
### *Spatial distribution of rabies in aardwolves and bat-eared foxes in the Northern Cape between 1994 and 2017*

Similarly, spatial analysis of the rabies-positive cases in aardwolves revealed 64% ( $n = 19$ ) more cases clustered in the western region of the Northern Cape Province with the majority of cases being confirmed between 2011 and 2017 (Figure 3).

Furthermore, other areas reported 36% ( $n = 11$ ) rabies-positive cases in the aardwolf between 2011 and 2017.

### *Molecular analysis and inferences on the viruses isolated from bat-eared foxes and aardwolves during the study period*

All the selected rabies viruses were successfully amplified and yielded an expected amplicon of approximately 850 bp in size (data not shown). The phylogenetic analysis revealed three main distinct viral clades (A, B and C) supported by a high posterior probability of 1 (Figure 4). Clade A comprised of rabies viruses obtained from both aardwolves ( $n = 9$ ) and bat-eared foxes ( $n = 6$ ) originating from different geographical areas in the eastern and northern regions of the Northern Cape Province indicating a single epidemiological rabies cycle (Figure 4, Table I). The pairwise mean distance of the viruses within Clade A was 98% nucleotide sequence similarity (Table I). The viral isolates were obtained between the year 1994 and 2017 (Table I). Clade B consisted of 18 RABV from central and eastern regions of the Northern Cape Province as compared to Clade A. At least 67% ( $n = 12$ ) of viral isolates were obtained from the aardwolves and 33% ( $n = 6$ ) were obtained from the bat-eared foxes. The data further shows that the viruses had 98% nucleotide sequence similarity on average (Table I). Clade C consisted of 50 RABV sequences obtained mainly from the western region of the Northern Cape Province ( $n = 38$ ) and neighbouring Western Cape Province ( $n = 12$ ) in the south. The data revealed that the viral isolates had 98% sequence identity, which indicate that they are from a single source of infection. Furthermore, Clade C could be delineated into Sub-clades C I and II with each sub-clade supported by a high posterior probability of 1 (Figure 4). The Sub-clade C I consisted of viral isolates mainly obtained from the aardwolves with the exception of single isolate (676/16) obtained from the bat-eared foxes in 2016 (Figure 4). The data analysis showed that the viral sequences in this Sub-clade C I had 99% sequence similarity on average indicating a single source of infection (Table I). The data analysis further showed that the most recent viral isolates obtained between 2015 and 2017 from the aardwolves had 100% nucleotide sequence identity. On the



**Figure 3:** The distribution map of rabies-positive cases confirmed in the aardwolf and the bat-eared fox between 1994 and 2017. The map was constructed using ArcGIS Software version 10.8.1.

other hand, Sub-clade C II consisted of viral isolates obtained from the bat-eared foxes from the Western Cape Province (Figure 4). The pairwise mean distances of the viruses within Sub-clade C II showed 99% sequence identity on average (Table I). The data analysis showed that the pairwise distance between Sub-clades C I and II had a 98% sequence identity on average (Table I).

### Discussion and conclusion

We investigated the molecular epidemiology of rabies in two wildlife species, the aardwolf and the bat-eared fox, both found in the dry and ecologically common areas of the Northern Cape Province of South Africa. The data presented here suggest two rabies virus (RABV) epidemiological cycles occurring within the aardwolf population perpetuated by the aardwolf-to-aardwolf and aardwolf-to-bat-eared fox transmissions and vice versa. In particular, the phylogenetic data demonstrated that the RABV isolates from the aardwolf clustered independently

from those obtained from the bat-eared fox, especially those originating from the western region of the Northern Cape Province. The heterogeneous clustering of the rabies viruses obtained from the aardwolf indicates that the RABV canid variant has been circulating in the aardwolf population for some time and, therefore, it appears to be well adapted to this wildlife species. A previous study demonstrated that the RABV was maintained by the Namibian kudu (*Tragelaphus strepsiceros*) population independently from the black-backed jackal species in similar temporal and ecological conditions (Scott et al. 2013). Another study reported an unusually high transmission of the raccoon (*Procyon lotor*) rabies variant to skunks (*Mephitis spp.*), demonstrating that the virus had already undergone a host shift into the skunk population (Wallace et al. 2014). Even though spillover events from RABV maintenance host species into other species are common, most do not result in ongoing transmission or host shifts (Mollentze et al. 2020). Furthermore, the study

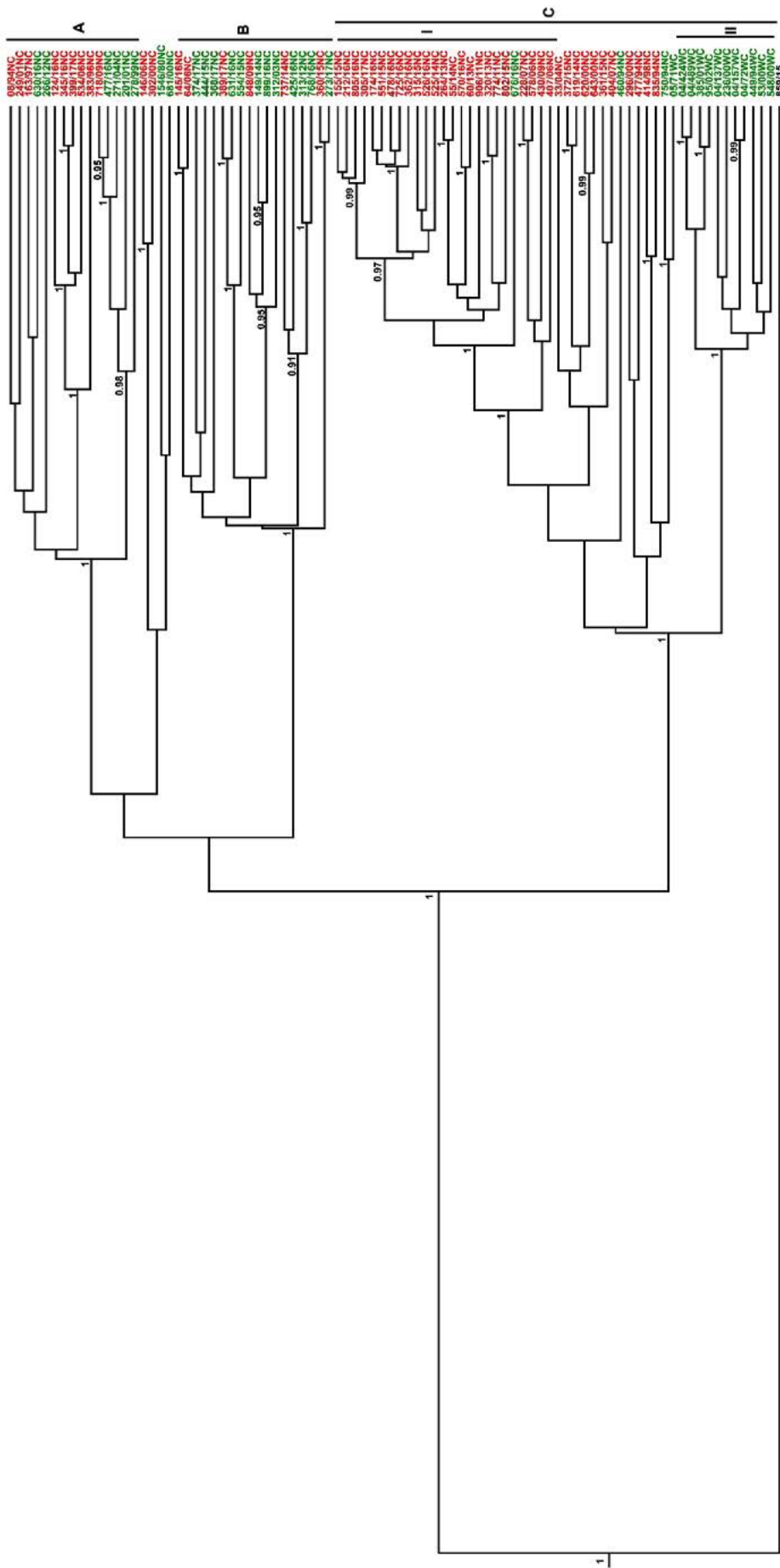


Figure 4: Maximum clade credibility tree illustrating the genetic relationships amongst the rabies virus isolates obtained from the aardwolves (red) and bat-eared foxes (green) obtained from the Northern and Western Cape provinces of South Africa. The horizontal branch lengths are proportional to the similarity of the sequences and mongoose rabies virus variant (859/15) was used to root the tree. Note: NC denotes Northern Cape Province bat-eared fox and WC denotes Western Cape Province bat-eared fox respectively. The letters A, B and C represent three main clades whereas symbols C I and II represent the sub-clades.

suggested that the RABV transmission into new species with a warmer body temperature than the current maintenance host is more likely to become established as ongoing transmissions and/or maintenance host species (Mollentze et al. 2020).

The data presented suggest a common source of RABV infection in the aardwolf population especially for the viruses obtained between 2015 and 2017 in the western region of the Northern Cape Province. The data revealed that the monophyletic clade from the viral isolates from the western region of the Northern Cape Province shared a common and recent ancestor with the RABV isolates from the bat-eared fox from the Western Cape Province, which suggests cross species transmission events between the two species. A similar phenomenon was observed in Namibia where kudu rabies resulted from spillover infection from black-backed jackals and established itself as an independent rabies epidemiological cycle within the kudu population (Scott et al. 2013). Therefore, it appears that the aardwolf has been instrumental in maintaining and transmitting the RABV canid variant infection in this geographical area of the country. Our data suggest the aardwolf-associated RABV canid variant has most likely evolved in an independent epidemiological cycle originating from the bat-eared fox probably during long-term repeated infection in this part of the country. This could have resulted from sharing of limited dietary resources by these insectivorous carnivores particularly during the dry winter season and/or use of the same dens, as aardwolves tend to use dens of similar sized carnivores (Anderson 1994; Richardson 1987). It is evident that there is an ease of exchange of the RABV canid variant amongst the wild insectivorous carnivores in the Western Cape and Northern Cape Provinces, which warrants further investigation. We do not have a plausible explanation for the decline in the number of recorded rabies-positive cases in aardwolves between 2016 and 2017 and this may warrant field studies and further investigation. In the Caribbean Islands, domestic dogs and mongooses maintain the same dog-derived RABV and both act as maintenance hosts (Nadin-Davies et al. 2008; Velasco Villa et al. 2017). The presence of multiple maintenance host species could have an impact on the control measures and re-emergence of the disease in a particular geographic area. Therefore, understanding the involvement of various maintenance host species in the epidemiology of rabies can add value to the formulation of a rabies control strategy in the region.

The recent passive surveillance data showed that aardwolves accounted for the majority of rabies-positive cases in the Northern Cape Province (ARC-OVR, unpublished records) and this observation, together with phylogenetic analysis undertaken here, lends support to a maintenance and transmission role of the RABV canid variant in this species. Similar observations from epidemiological and surveillance data in south-east China suggested that ferret badger (*Melogale moschata*)-associated rabies has likely formed as an independent cycle originating from dogs during the long-term rabies infection (Liu et al. 2010). However, passive surveillance programmes only rely on samples submitted to the laboratory for confirmation, which may result in a significant bias (i.e. not all suspected rabies samples are submitted to the laboratory for confirmation). On the contrary,

implementation of passive surveillance in Brazil resulted in an increase in the number of samples submitted for rabies testing and the frequency of positive samples significantly (Duarte et al. 2020). It was found that the majority of the aardwolf-associated rabies virus cases occurred in the sheep and goat farming areas of the Northern Cape Province where one farmer alerted the veterinary officials to the deaths occurring in this species. Since the aardwolves do not prey on livestock, most farmers are not concerned about their presence on their farms and consequently do not report any morbidities and mortalities from this species occurring on their respective farms (personal communication with a farmer). Therefore, such behaviour would result in a substantial underestimation of the actual incidence of the aardwolf-associated rabies virus cases in this area and elsewhere in the country.

The passive surveillance data reported in this study revealed that the majority of rabies-positive cases occurred in the colder months peaking in July and a similar pattern was observed in the bat-eared foxes, which peaked during the dry season (Thomson & Meredith 1993). A previous study demonstrated that during winter months the food sources for the aardwolf become scarce and this wildlife carnivore species switches to a different diet, a termite species called *Hodotermes mossambicus*, which makes up 90% of the bat-eared fox diet (Richardson 1985). It could, therefore, be speculated that during the winter periods, scarcity of food could potentially lead to possible intra- or inter-species contacts, as these wildlife hosts search for food or food competition, which can promote cross-species rabies virus transmission between the two species. Aggressive interaction during the territorial defense and breeding season could also create an ideal opportunity for intra- or inter-species transmission of the rabies virus amongst the population in the area. It was reported that bite wounds sustained through African wild dog (*Lycaon pictus*) fights with other pack members and excessive licking of wounds by sick animals could enhance the direct transmission of RABV infections within a population (Canning et al. 2019). Furthermore, the wide-ranging movement of these wild animals together with sharing of food sources during colder months might facilitate close contact between remote individuals in the population (McKenzie 1993).

The existence of rabies epidemiological cycles involving both the aardwolf and the bat-eared fox demonstrates that the RABV is easily exchanged between the two species especially in the eastern region of the Northern Cape Province. Findings from a previous study showed that the bat-eared fox is responsible for the maintenance and transmission of the rabies virus in this region of South Africa (Sabeta et al. 2007) and the data presented here suggest that a cross-species transmission event occurred from the bat-eared foxes into the aardwolf population. Such phenomena of cross-species transmission events result in enhancing the diversity of RABVs and increase the chance of virus transmission between different canid groups (Carnieli Jr 2006). This study provides some evidence that the aardwolves are capable of sustaining RABV infection cycles independent of the bat-eared foxes in this geographical area of South Africa. Despite a general lack of population statistics for the aardwolf, it is believed that this species has a stable population in the



region (Green 2015) and this may allow the RABV infection to be maintained in this population. Surveillance data of the aardwolf-associated rabies virus cases in the Northern Cape Province over the last two decades together with a detailed phylogenetic analysis presented here demonstrates a RABV host shift from the bat-eared fox and the spread of the RABV in the aardwolf population. The RABV transmission dynamics are poorly understood in wildlife populations in South Africa. These results may be useful and contribute to future wildlife rabies control strategies in the Northern Cape Province. Understanding the role of different species in the transmission of multi-host pathogens, such as RABV, is vital for effective control strategies. Improved wildlife rabies virus surveillance and control strategies are required to ensure effective control of rabies in the relevant territories.

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### Conflict of interest

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