Emerging epidemic dog rabies in coastal South Africa: A molecular epidemiological analysis

Peter Coetzee[a] **and Louis H. Nel** [a]

[a]Department of Microbiology and Plant Pathology, **University of Pretoria**, 0001 Pretoria, South Africa

Abstract

Towards understanding the molecular epidemiology of a severe dog rabies epidemic in the KwaZulu Natal province of South Africa, we analyzed a variable 592 nucleotide genome sequence domain of 170 rabies viruses from KwaZulu Natal and surrounding regions. Viruses from the KwaZulu Natal and Eastern Cape provinces belonged to a unique lineage, circulating as two independent and expanding epidemiological cycles. The first presented as closely related dog cycles along the eastern coastal regions of the two provinces, while the second, in northern KwaZulu Natal, has entered into at least one wildlife reservoir, the black backed jackal. We underline the success and opportunism of rabies in southern Africa, in a likely reflection of the emergence and radiation of rabies in new host species and locales throughout the larger continent as a whole.

Article Outline

1. Introduction

Rabies virus (RABV) is one of the most widespread animal viruses and continues to scourge virtually the entire globe as the agent of severe and lethal encephalopathy of all terrestrial mammals, including man (Rupprecht et al., 2002, Swanepoel, 2005 and WHO, 2004). RABV belongs to genotype 1 of the *Lyssavirus* genus of the family *Rhabdoviridae*, order *Mononegavirales* and contains a single negative sense RNA genome of approximately 12 kb. In southern Africa, two biotypes of RABV are maintained independently among the members of the *Herpestidae* and *Canidae* families, respectively (Von Teichman et al., 1995 and Nel et al., 2005). Canid rabies is relatively new to the southern African subcontinent, having been introduced from infectious cycles that had existed among domestic dogs in Angola in the 1940s (Swanepoel, 2005). It has since spread widely throughout the Republic of South Africa (RSA), with the increasing involvement of wildlife, including bat eared foxes (*Otocyon megalotis*) in the western regions of the country, and black backed jackal (*Canis mesomelas*) in the northern Limpopo province (Bishop et al., 2003, Sabeta et al., 2003 and Swanepoel, 2005). However, in recent decades, the majority of animal and human rabies cases in RSA consistently occurred in the province of KwaZulu Natal (KZN), where the disease has become endemic in the large population of domestic dogs (*Canis familiaris*) of this province (Bishop et al., 2003 and Swanepoel, 2005). There is a close correlation between the number of cases in dogs, with those in humans and livestock, and the control of dog rabies remains the single most important factor in minimizing the public and veterinary health consequences of rabies here, as it is throughout Africa and in most of the rest of the developing world.

KZN is located on the eastern seaboard of RSA, and is one of the smallest (92,100 km^2) and the most populated (approximately 9.5 million people) of the nine RSA provinces. It extends from the international borders with Swaziland and Mozambique in the north, to

the province of the Eastern Cape (EC) in the south, while inland it is bound by the provinces of the Freestate and Mpumalanga, and by the Kingdom of Lesotho (Fig. 1). Two dog rabies epidemics, believed to have originated from dog endemic regions which had existed in southern Mozambique since 1952, have occurred among domestic dogs in KZN (Bishop et al., 2003 and Swanepoel, 2005). The first of these epidemics started in 1964, and apparently without spreading into the adjacent EC, ended by 1968, due to mass vaccination and strict dog control (Bishop et al., 2003 and Swanepoel, 2005). The second epidemic, which started in 1976, coincided with the outbreak of civil war in Mozambique and the fleeing of refugees across the international border with RSA (KZN). This epidemic has proven to be intractable despite the concerted control efforts of the South African Directorate of Veterinary Services (Bishop et al., 2003 and Swanepoel, 2005) and is thought to have penetrated far and wide into the province and adjacent regions—rabies has for example been reported from the EC since 1987 (Bishop et al., 2003 and Swanepoel, 2005). Reasons for the persistence of the second epidemic are varied, but of significant importance is an increase in urbanization since the late 1980's, mostly due to social and political changes. The development of numerous informal settlements around towns and cities followed and large dog populations capable of maintaining the disease were rapidly introduced (Kloeck, 1993). Subsequent logistical, financial and managerial difficulties in the control of dog movement and in the implementation and maintenance of successful vaccination strategies, contribute to the persistence of the epidemic (Randles, 2003 and Perret, 2005).

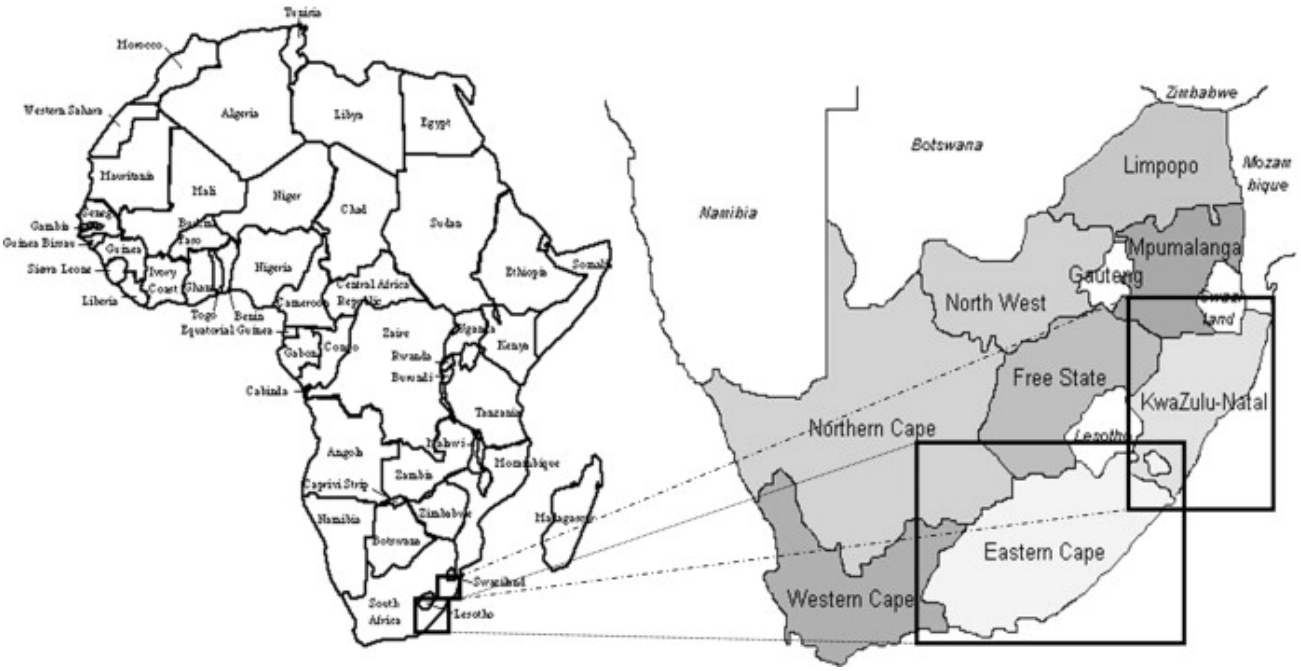

Fig. 1. Map of Africa and South Africa indicating the geographic location of the KwaZulu Natal and Eastern Cape provinces.

A better understanding of the molecular epidemiology of this growing rabies epidemic should assist in future surveillance and control efforts and towards this objective, we conducted a molecular sequence analysis of a representative panel of viruses, obtained from the KZN province during a given calendar year (2003). Previous molecular epidemiological studies in southern Africa included a small number of isolates from KZN, but were primarily aimed at illustrating the distinction between the canid and mongoose biotypes of the disease (King, 1993, Von Teichman et al., 1995 and Nel et al., 1997), or were focused on determining the genetic diversity of the canid biotype over a broader geographical region (Sabeta et al., 2003). Here our emphasis was placed on determining the regional genetic variation of RABV within KZN, and on delineating the course of the epidemic through the different magisterial districts of the province. Subsequently, we assessed the relationship of this epidemic with cycles from elsewhere in southern Africa. Our analysis was based on the carboxyl terminal domain of the glycoprotein (G, cytoplasmic domain) and the G-L intergenic region (L representing the downstream ‘large’ viral polymerase gene), which constitute the most variable portion of

the RABV genome (Tordo and Kouknetzoff, 1993). This target was considered appropriate for distinguishing closely related viral variants from each other, as it has been shown in various studies to be well suited for the investigation of the molecular epidemiology of rabies in defined geographical domains (Tordo et al., 1986, Sacramento et al., 1991, Nel et al., 1993, Nadin-Davis, 2000 and Paez et al., 2003).

In this study we present the first molecular epidemiological evidence for the emerging distribution patterns of rabies in coastal southern Africa and resolve an epidemiological scenario that may well be typical for much of the African continent.

2. Materials and methods

2.1. Viruses

Details of viruses analyzed are presented in Table 1. During the year 2003, 235 animals from KZN were diagnosed as rabies positive with a standard immunofluorescent assay (Bishop et al., 2003). Samples of infected brain material were stored at −20 °C in 50% glycerol-phosphate buffered saline (PBS) without further passage (Allerton Veterinary Laboratories, Pietermaritzburg, RSA). Of these, 123 samples that represented each of the affected magisterial districts of KZN, were selected for further analysis. Also included were 19 samples from animal cases in EC (Onderstepoort Veterinary Research Institute, Pretoria, RSA).

Table 1.

Panel of rabies viruses from the KwaZulu Natal (KZN) and the Eastern Cape (EC) provinces of South Africa

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
1	KZNcw03.83	Bovine	South Africa/KwaZulu Natal	Alfred	2003	G17	**DQ841424**
2	KZNdg03.89	Canine	South Africa/KwaZulu Natal	Pongola	2003	L4	**DQ841427**
3	KZNcw03.103	Bovine	South Africa/KwaZulu Natal	Pongola	2003	L4	**DQ841425**
4	KZNdg03.105	Canine	South Africa/KwaZulu Natal	Eshowe	2003	L10	**DQ841428**
5	KZNdg03.106	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	O9	**DQ841429**
6	KZNdg03.114	Canine	South Africa/KwaZulu Natal	Mount Currie	2003	D17	**DQ841430**
7	KZNdg03.120	Canine	South Africa/KwaZulu Natal	Alfred	2003	H17	**DQ841431**
8	KZNdg03.121	Canine	South Africa/KwaZulu Natal	Alfred	2003	G17	**DQ841432**
9	KZNdg03.127	Canine	South Africa/KwaZulu Natal	Ixopo	2003	G15	**DQ841433**
10	KZNdg03.129	Canine	South Africa/KwaZulu Natal	Inanda	2003	K13	**DQ841434**
11	KZNdg03.133	Canine	South Africa/KwaZulu Natal	Lower Tugela	2003	L12	**DQ841435**
12	KZNdg03.137	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	P7	**DQ841436**
13	KZNdg03.142	Canine	South Africa/KwaZulu Natal	Durban	2003	K14	**DQ841437**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
14	KZNdg03.147	Canine	South Africa/KwaZulu Natal	Umzinto	2003	I17	**DQ841438**
15	KZNdg03.149	Canine	South Africa/KwaZulu Natal	Umvoti	2003	I11	**DQ841439**
16	KZNdg03.152	Canine	South Africa/KwaZulu Natal	Ndwedwe	2003	J13	**DQ841440**
17	KZNdg03.169	Canine	South Africa/KwaZulu Natal	Eshowe	2003	L10	**DQ841441**
18	KZNdg03.170	Canine	South Africa/KwaZulu Natal	Vryheid	2003	K6	**DQ841442**
19	KZNdg03.176	Canine	South Africa/KwaZulu Natal	Vryheid	2003	K6	**DQ841443**
20	KZNdg03.180	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	P6	**DQ841444**
21	KZNdg03.192	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H18	**DQ841445**
22	KZNdg03.194	Canine	South Africa/KwaZulu Natal	Lower Tugela	2003	K12	**DQ841446**
23	KZNdg03.200	Canine	South Africa/KwaZulu Natal	Lower Tugela	2003	L12	**DQ841447**
24	KZNDg03.204	Canine	South Africa/KwaZulu Natal	Lower Tugela	2003	L11	**DQ841448**
25	KZNdg03.205	Canine	South Africa/KwaZulu Natal	Pietemaritzburg	2003	H13	**DQ841449**
26	KZNdg03.209	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841450**
27	KZNdg03.213	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H17	**DQ841451**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
28	KZNdg03.214	Canine	South Africa/KwaZulu Natal	Pongola	2003	L4	**DQ841452**
29	KZNdg03.215	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841453**
30	KZNdg03.225	Canine	South Africa/KwaZulu Natal	Umzimkulu	2003	E16	**DQ841454**
31	KZNdg03.230	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841455**
32	KZNdg03.235	Canine	South Africa/KwaZulu Natal	Inanda	2003	J13	**DQ841456**
33	KZNdg03.236	Canine	South Africa/KwaZulu Natal	Alfred	2003	F17	**DQ841457**
34	KZNdg03.237	Canine	South Africa/KwaZulu Natal	Ndwedwe	2003	J13	**DQ841458**
35	KZNdg03.241	Canine	South Africa/KwaZulu Natal	Mhlabatini	2003	L8	**DQ841459**
36	KZNdg03.247	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841460**
37	KZNdg03.251	Canine	South Africa/KwaZulu Natal	Ndwedwe	2003	J13	**DQ841461**
38	KZNdg03.253	Canine	South Africa/KwaZulu Natal	Vyheid	2003	J6	**DQ841462**
39	KZNdg03.254	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841463**
40	KZNdg03.256	Canine	South Africa/KwaZulu Natal	Pinetown	2003	J14	**DQ841464**
41	KZNdg03.263	Canine	South Africa/KwaZulu Natal	Inanda	2003	J13	**DQ841465**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
42	KZNdg03.264	Canine	South Africa/KwaZulu Natal	Ngotshe	2003	L5	**DQ841466**
43	KZNdg03.265	Canine	South Africa/KwaZulu Natal	Pinetown	2003	J14	**DQ841464**
44	KZNdg03.269	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J5	**DQ841468**
45	KZNdg03.270	Canine	South Africa/KwaZulu Natal	Eshowe	2003	M10	**DQ841469**
46	KZNdg03.276	Canine	South Africa/KwaZulu Natal	Nwedwe	2003	J13	**DQ841470**
47	KZNdg03.290	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841471**
48	KZNdg03.292	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841472**
49	KZNdg03.293	Canine	South Africa/KwaZulu Natal	Durban	2003	J14	**DQ841473**
50	KZNdg03.299	Canine	South Africa/KwaZulu Natal	Durban	2003	K14	**DQ841474**
51	KZNdg03.302	Canine	South Africa/KwaZulu Natal	Eshowe	2003	L10	**DQ841475**
52	KZNdg03.306	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841476**
53	KZNdg03.307	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841477**
54	KZNdg03.308	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841478**
55	KZNdg03.309	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841479**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
56	KZNdg03.314	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O8	**DQ841480**
57	KZNdg03.315	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841481**
58	KZNdg03.316	Canine	South Africa/KwaZulu Natal	Nkandhla	2003	J9	**DQ841482**
59	KZNdg03.321	Canine	South Africa/KwaZulu Natal	Ngotshe	2003	L5	**DQ841483**
60	KZNdg03.326	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841484**
61	KZNdg03.328	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	M4	**DQ841485**
62	KZNgt03.330	Caprine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841542**
63	KZNdg03.335	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J5	**DQ841486**
64	KZNdg03.336	Canine	South Africa/KwaZulu Natal	?	2003	?	**DQ841487**
65	KZNdg03.340	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841488**
66	KZNdg03.343	Canine	South Africa/KwaZulu Natal	Mhlabatini	2003	L7	**DQ841489**
67	KZNgt03.358	Caprine	South Africa/KwaZulu Natal	Mhlabatini	2003	M7	**DQ841543**
68	KZNdg03.359	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841490**
69	KZNdg03.360	Canine	South Africa/KwaZulu Natal	Pongola	2003	M4	**DQ841491**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
70	KZNgt03.364	Caprine	South Africa/KwaZulu Natal	Pongola	2003	L4	**DQ841544**
71	KZNdg03.366	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H18	**DQ841492**
72	KZNdg03.368	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	O9	**DQ841493**
73	KZNdg03.375	Canine	South Africa/KwaZulu Natal	Eshowe	2003	J6	**DQ841494**
74	KZNdg03.378	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H17	**DQ841495**
75	KZNdg03.382	Canine	South Africa/KwaZulu Natal	Ixopo	2003	G15	**DQ841496**
76	KZNdg03.387	Canine	South Africa/KwaZulu Natal	Durban	2003	J14	**DQ841497**
77	KZNdg03.389	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841498**
78	KZNdg03.391	Canine	South Africa/KwaZulu Natal	Umzinto	2003	I16	**DQ841499**
79	KZNdg03.399	Canine	South Africa/KwaZulu Natal	Umzinto	2003	I16	**DQ841500**
80	KZNdg03.400	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841501**
81	KZNdg03.404	Canine	South Africa/KwaZulu Natal	Ixopo	2003	G15	**DQ841502**
82	KZNdg03.406	Canine	South Africa/KwaZulu Natal	Inanda	2003	K13	**DQ841503**
83	KZNdg03.407	Canine	South Africa/KwaZulu Natal	Ubombo	2003	O5	**DQ841504**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
84	KZNdg03.409	Canine	South Africa/KwaZulu Natal	Mount Currie	2003	E17	**DQ841505**
85	KZNdg03.410	Canine	South Africa/KwaZulu Natal	Eshowe	2003	L10	**DQ841506**
86	KZNdg03.411	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841507**
87	KZNdg03.417	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841508**
88	KZNdg03.418	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H17	**DQ841509**
89	KZNdg03.425	Canine	South Africa/KwaZulu Natal	Vryheid	2003	L6	**DQ841510**
90	KZNdg03.430	Canine	South Africa/KwaZulu Natal	Nongoma	2003	N6	**DQ841511**
91	KZNdg03.431	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O7	**DQ841512**
92	KZNshp03.433	Ovine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841403**
93	KZNdg03.437	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	O9	**DQ841513**
94	KZNdg03.453	Canine	South Africa/KwaZulu Natal	Durban	2003	J14	**DQ841514**
95	KZNdg03.454	Canine	South Africa/KwaZulu Natal	Nongoma	2003	M5	**DQ841515**
96	KZNdg03.455	Canine	South Africa/KwaZulu Natal	Vryheid	2003	K7	**DQ841516**
97	KZNdg03.461	Canine	South Africa/KwaZulu Natal	Lower Tugela	2003	K12	**DQ841517**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
98	KZNdg03.463	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841518**
99	KZNcw03.467	Bovine	South Africa/KwaZulu Natal	Ubombo	2003	O5	**DQ841545**
100	KZNdg03.470	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841519**
101	KZNdg03.474	Canine	South Africa/KwaZulu Natal	Mount Currie	2003	D17	**DQ841520**
102	KZNdg03.475	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841521**
103	KZNdg03.478	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841522**
104	KZNdg03.485	Canine	South Africa/KwaZulu Natal	Mapumulo	2003	K11	**DQ841523**
105	KZNdg03.491	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H17	**DQ841524**
106	KZNdg03.492	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H17	**DQ841525**
107	KZNdg03.494	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	O9	**DQ841526**
108	KZNdg03.502	Canine	South Africa/KwaZulu Natal	Port Shepstone	2003	H16	**DQ841527**
109	KZNdg03.503	Canine	South Africa/KwaZulu Natal	Ingwavuma	2003	Q3	**DQ841528**
110	KZNdg03.507	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841529**
111	KZNdg03.509	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841530**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
112	KZNdg03.510	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841531**
113	KZNdg03.513	Canine	South Africa/KwaZulu Natal	Eshowe	2003	L10	**DQ841532**
114	KZNdg03.514	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841533**
115	KZNdg03.518	Canine	South Africa/KwaZulu Natal	Ubombo	2003	P6	**DQ841534**
116	KZNdg03.568	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841535**
117	KZNdg03.583	Canine	South Africa/KwaZulu Natal	Vryheid	2003	J6	**DQ841536**
118	KZNdg03.588	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	P7	**DQ841537**
119	KZNdg03.589	Canine	South Africa/KwaZulu Natal	Vryheid	2003	K7	**DQ841538**
120	KZNdg03.594	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	N9	**DQ841539**
121	KZNcw03.620	Bovine	South Africa/KwaZulu Natal	Port Shepstone	2003	H17	**DQ841426**
122	KZNdg03.621	Canine	South Africa/KwaZulu Natal	Hlabisa	2003	O8	**DQ841540**
123	KZNdg03.672	Canine	South Africa/KwaZulu Natal	Lower Umfolozi	2003	O10	**DQ841541**
124	ECdg03.05	Canine	South Africa/Eastern Cape	Elliott	2003	*	**DQ841404**
125	ECdg03.28	Canine	South Africa/Eastern Cape	Lupapasi	2003	*	**DQ841406**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
126	ECdg03.91	Canine	South Africa/Eastern Cape	Haga Haga, Everest	2003	*	**DQ841408**
127	ECdg03.179	Canine	South Africa/Eastern Cape	Elliott	2003	*	**DQ841410**
128	ECdg03.180	Canine	South Africa/Eastern Cape	Elliott	2003	*	**DQ841411**
129	ECdg03.336	Canine	South Africa/Eastern Cape	Bayview	2003	*	**DQ841413**
130	ECdg03.377	Canine	South Africa/Eastern Cape	Lower Nququ	2003	*	**DQ841415**
131	ECdg03.567	Canine	South Africa/Eastern Cape	Ncanaseni	2003	*	**DQ841418**
132	ECdg03.730	Canine	South Africa/Eastern Cape	Ncambedlana	2003	*	**DQ841419**
133	ECdg03.751	Canine	South Africa/Eastern Cape	Vidgesville	2003	*	**DQ841420**
134	ECdg03.779	Canine	South Africa/Eastern Cape	All Saints	2003	*	**DQ841421**
135	ECdg03.936	Canine	South Africa/Eastern Cape	Butterworth	2003	*	**DQ841422**
136	ECdg04.25	Canine	South Africa/Eastern Cape	Ngangelizwe	2004	*	**DQ841405**
137	ECdg04.43	Canine	South Africa/Eastern Cape	Upper Ngqwaba	2004	*	**DQ841407**
138	ECdg04.111	Canine	South Africa/Eastern Cape	Centuli Location	2004	*	**DQ841409**
139	ECdg04.218	Canine	South Africa/Eastern Cape	North Crest	2004	*	**DQ841412**

Virus number	Allerton reference number[a]	Species[b]	Country/province	Magisterial district	Year of isolation	Grid reference number[c]	Genbank accession number
140	ECdg04.376	Canine	South Africa/Eastern Cape	Sterkspruit	2004	*	**DQ841414**
141	ECdg04.377	Canine	South Africa/Eastern Cape	Ugie	2004	*	**DQ841416**
142	ECdg04.499	Canine	South Africa/Eastern Cape	Barkley East	2004	*	**DQ841417**

[a] Lab reference numbers: viruses from the Allerton Regional Veterinary Laboratory (KZN) are named using the zero-three or zero-four designation.

[b] We use the term 'canine' to refer to domestic dogs (*C. familiaris*).

[c] The approximate regions from where isolates were obtained, were indicated on a map of the KwaZulu Natal province by using a grid system, as implemented on the isolate submission forms of the Allerton Regional Veterinary Laboratory (KZN). Similar grid reference numbers were not available for isolates from the Eastern Cape.

2.2. cDNA synthesis, PCR amplification and sequencing

Total RNA was extracted from infected brain material with Trizol reagent (Promega) and cDNA synthesized as described previously (Von Teichman et al., 1995 and Sabeta et al., 2003). PCR was performed (GeneAmp PCR 2700) in a 50 µl reaction consisting of 1 µl cDNA, 5 µl 10X PCR buffer (Promega), 100 µM each of the four dNTPs (Promega), 10 pmol each of the G (+) and L (−) primers, and 1 U Taq DNA polymerase (Promega). The primers (Integrated DNA Technologies, RSA) have been described by Sacramento et al. (1991). The PCR cycle reaction was as follows: 2 min, 94 °C; 30 cycles of 94 °C/50s, 45 °C/90s, 72 °C/60s; 7 min, 72 °C. Amplicons were analyzed by agarose gel electrophoresis and purified with a Wizard® SV kit (Promega). Cycle sequencing reactions were performed with the V 3.1 BigDye Terminator system (PE Applied Biosystems) together with the G (+) primer, after which unincorporated labeled ddNTPs were removed by ethanol precipitation. The reactions were resolved on an ABI 3100 DNA sequencer and sequences were trimmed with the Bioedit V.7.0 sequence alignment editor (Hall, 1999). All sequences were submitted to an international sequence repository (Genbank accession numbers; Table 1).

2.3. Phylogenetic analysis

Analyses were based on the continuous 592-nucleotide sequences, aligned with CLUSTALW (Thompson et al., 1994), as a subroutine of the Bioedit sequence alignment editor (Hall, 1999). Genetic distances were calculated with Kimura's two parameter method (MEGA3; Kumar et al., 2004), and used for the construction of two neighbourhood-joining (NJ) trees by the method of Saitou and Nei (1987). Confidence values for the tree topologies were evaluated with a bootstrap analysis of 1000 pseudoreplicate datasets, and considered meaningful for phylogenetic groupings when above 70% (Hills and Bull, 1993). The results were validated by maximum likelihood analysis (Swofford and Olsen, 1990).

3. Results

The vast majority of the 235 positive rabies cases identified in 2003 in KZN occurred among domestic dogs (85%). These cases were not evenly distributed through the province, but were primarily localized in the more densely populated coastal regions. KZN is divided into 51 independently governed magisterial districts and it was convenient to use this divisioning system towards obtaining a representative virus sampling. We selected approximately half of the rabies virus samples from each magisterial district, arguing that this strategy would ensure sufficient representation from all of the affected areas of the province.

The G-L intergenic sequence target offered us satisfactory resolution, and it was found that the viruses from KZN shared an approximate intrinsic sequence identity of 98.9% (Kimura 2-parameter model, mean pairwise distance data not shown). All of these viruses were also closely related to other members of the RSA canid biotype, from elsewhere in southern Africa. However, on average the KZN viruses differed by 19.1% from the corresponding sequence of the PV genome, and by 29.4% from a classical mongoose biotype RABV isolate (m669.90; Nel et al., 2005). Comparable results were obtained with the inclusion of sequence data from the EC viruses (results not shown), and all viruses from KZN and EC were concluded to conform to the canid biotype (Von Teichman et al., 1995). Our viruses also lacked the first of two hypothetical transcription termination and polyadenylation signals, found to be present for the G gene of the ERA and PV genomes. The absence of this signal has previously been demonstrated for street viruses from Europe as well as from South Africa and Zimbabwe (Wunner, 2002 and Sabeta et al., 2003).

Two phylogenetic trees were constructed in order to address different aspects of this epidemic. The first (Fig. 2), was constructed with all the RABV sequences from KZN (n = 123), and was aimed at demonstrating the evolutionary relationships between viruses from the different magisterial districts of the province. The epidemiological data of the viruses are provided in Table 1 and the corresponding location of the rabies cases are shown in Fig. 4. Our phylogenetic analyses demonstrated the existence of two major

KZN RABV subfamilies, designated A and B. The division between these subfamilies was characterized by a mean nucleotide sequence divergence of 1.9%, and was supported by a bootstrap value of 100%. Subfamily A was by far the largest (composed of 102 viruses) and clearly represented the principal dog rabies cycle of KZN. Viruses belonging to this subfamily were primarily encountered in the eastern coastal districts of KZN, stretching from the border with southern Mozambique all along the coast to southern KZN. Subfamily A could be divided into two groups, the first of which (group I) consisted of seven geographically influenced sequence clusters (KZN/A/V1-KZN/A/V7, Fig. 2). Some of these clusters were also not monophyletic, in which case the subdivisions always corresponded to highly localized outbreaks of the disease (e.g. KZN/A/V3/cl1-cl2, Fig. 2). The second group of subfamily A (group II) consisted of a single sequence cluster, made up of only three viruses, found on the southernmost border of KZN with the EC province. Considering its phylogenetic and geographical distinction, this group was thought to represent a cycle distinct from the main KZN epidemic.

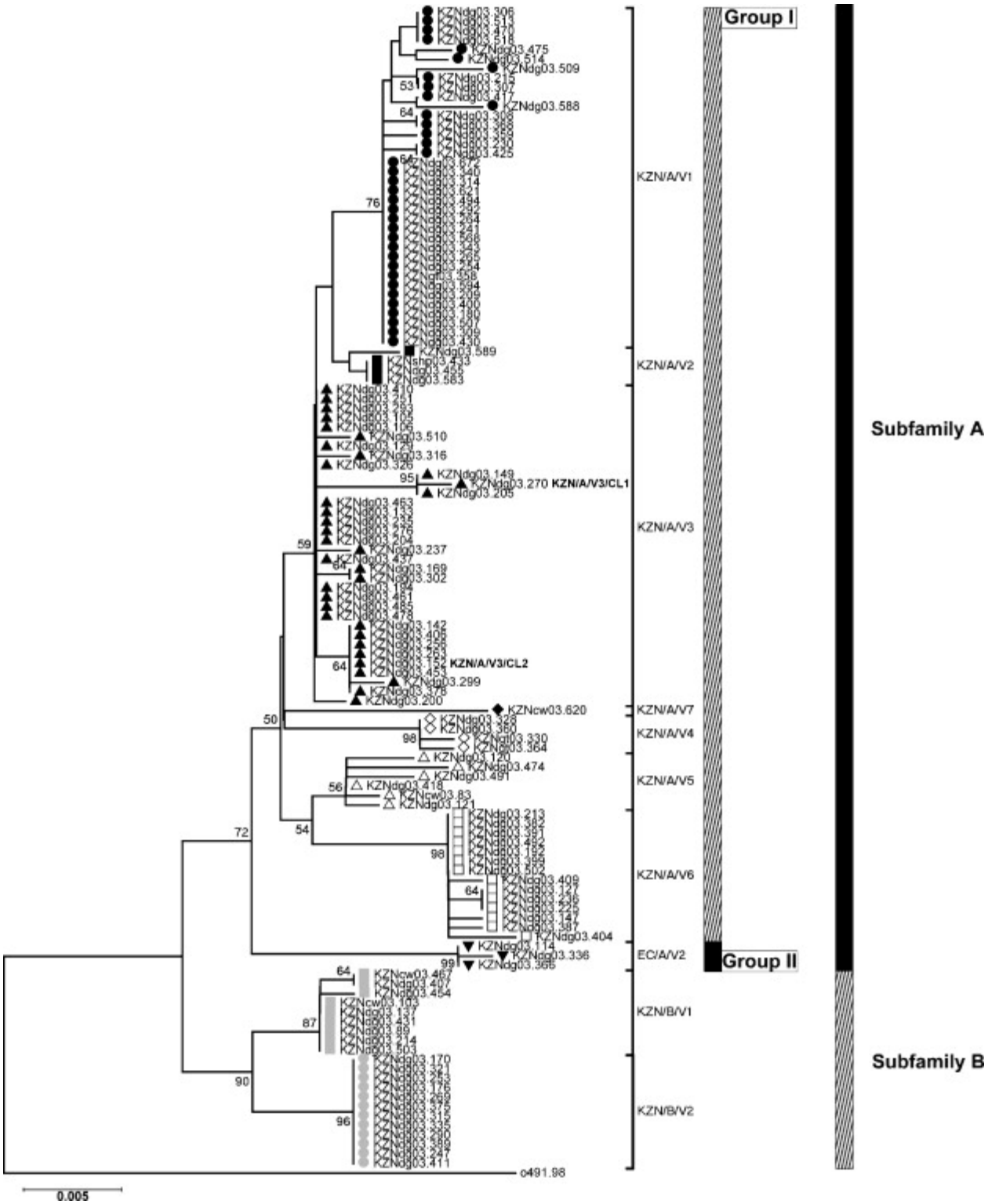

Fig. 2. Neighbourhood-joining tree of 123 nucleotide sequences of the cytoplasmic domain of the glycoprotein, and G-L intergenic region, for canine and domestic livestock

rabies viruses from KwaZulu Natal. Horizontal branch lengths are proportional to the similarity of the sequences within and between groups, with the scale indicating the amount of nucleotide sequence divergence in substitutions per site and the vertical lines being provided for purposes of clarity only. The cognate nucleotide sequence of a bat eared fox isolate (o491.98) was used as reference sequence to root the tree. Subfamily and group divisions are indicated as discussed in the text, with virus numbers being preceded by a prefix indicating the geographic region (KZN, KwaZulu Natal) as well as host species of isolation (dg, dog/cw, bovine/gt, caprine/o, bat eared fox).

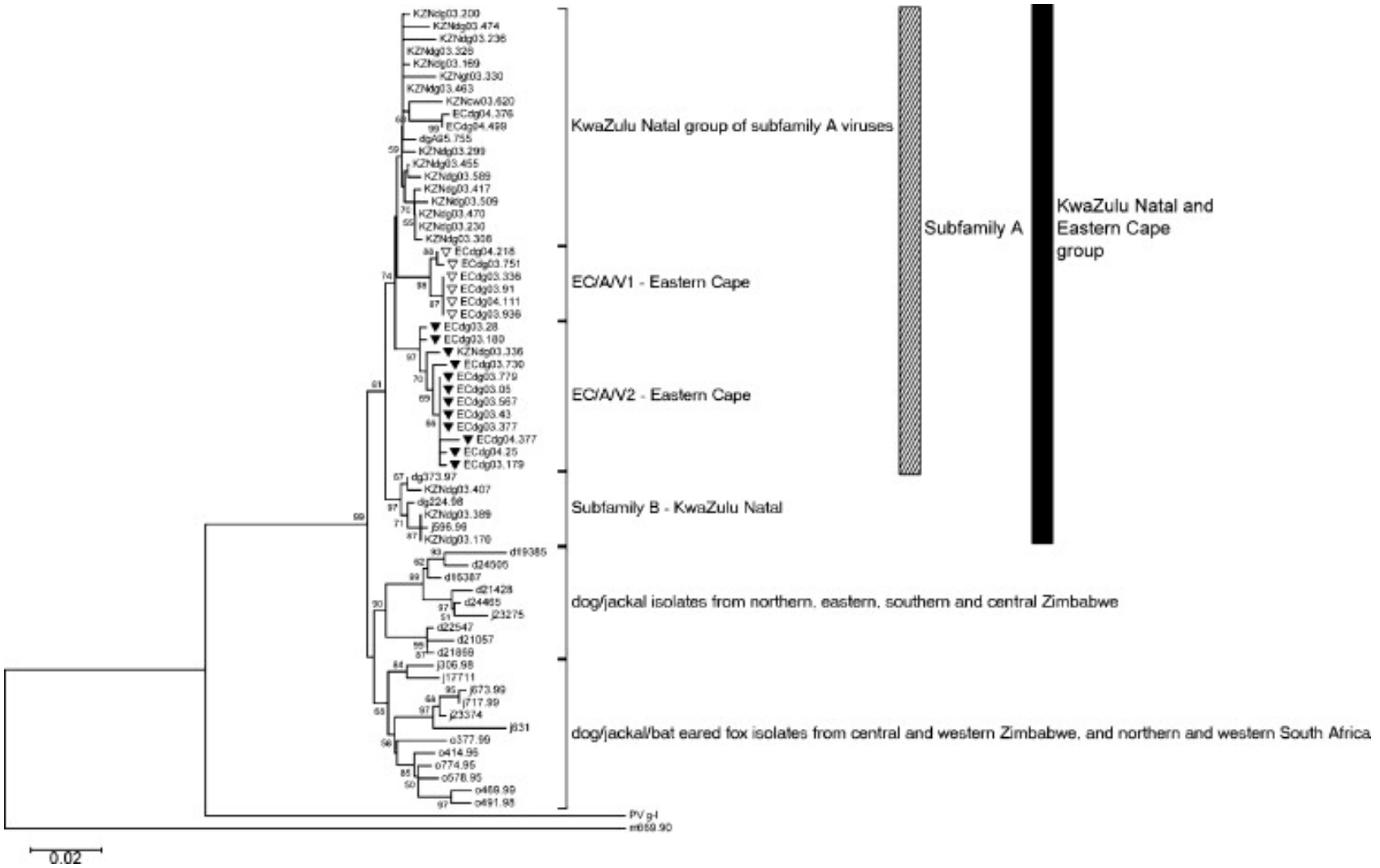

Fig. 3. Neighbourhood-joining tree of 64 nucleotide sequences of the cytoplasmic domain, and G-L intergenic region, for canine, domestic livestock and wildlife rabies viruses from KwaZulu Natal (n = 20), the Eastern Cape (n = 19), and rabies endemic regions

from elsewhere in South Africa and Zimbabwe (n = 25). Horizontal branch lengths are proportional to the similarity of the sequences within and between groups, with the scale indicating the amount of nucleotide sequence divergence in substitutions per site and the vertical lines being provided for purposes of clarity only. The cognate nucleotide sequences of the PV strain and a classical mongoose rabies virus isolate (m669.90) were used as reference sequences and to root the tree (**Tordo et al., 1986**, **Tordo et al., 1988** and **Von Teichman et al., 1995**). Isolate numbers are preceded by prefixes indicating the geographic region (KZN, KwaZulu Natal/EC, Eastern Cape) as well as host species of isolation (dg (d), dog/cw, bovine/gt, caprine/j, jackal/o, bat eared fox).

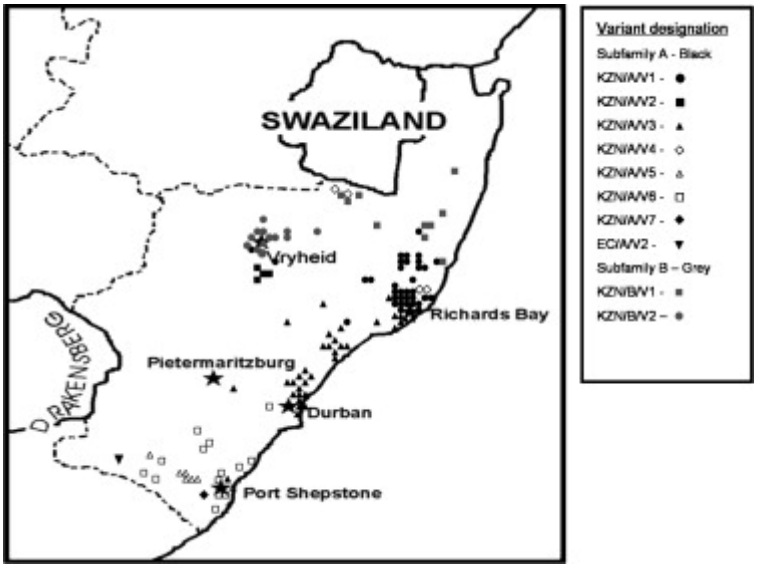

Fig. 4. A map of the KwaZulu Natal (KZN) province demonstrating the approximate geographic origin of rabies viruses that were sequenced during the course of this study. Symbols correspond to those that were used to indicate the respective viral groupings on the phylogenetic tree in **Fig. 2**.

The 21 viruses in subfamily B were very closely related (identity 99.5%), and were all encountered in the northern regions of the province bordering the southeastern Mpumalanga province and the international border with southern Swaziland (Fig. 4). This subfamily was nevertheless composed of two distinct clusters (KZN/B/V1-KZN/B/V2), representing localized outbreaks in this geographical domain (bootstrap value 90%, Fig. 2).

As a subsequent step in our study, we wanted to investigate the relationship of the rabies epidemic in KZN with the epidemiology of the disease in surrounding areas of South Africa. We also considered the role of wildlife canid species to be of particular importance in the larger epidemiological picture. Although rabies was first reported in 1893 from the neighbouring EC province, following an outbreak that was initiated by an infected dog imported from Britain, this outbreak was likely to have been eradicated without spillover into wildlife (Swanepoel, 2005). Since that outbreak, rabies incidence—if present in the EC, was not well documented, although the disease is known to have occurred with increasing frequency since at least 1987. Therefore, our next phylogenetic

analysis was based on selected viruses from KZN (n = 20), viruses from the adjacent province of the EC (n = 19) (Fig. 5), and reference RABV sequences (n = 25) that were obtained from canine rabies endemic regions from elsewhere in RSA and Zimbabwe (Table 2). It was apparent (Fig. 3) that the viruses from the KZN and EC provinces were, as a unique cluster, distinguishable from all the other RABV isolates included in this analysis. Within this KZN and EC group, the subfamilies A and B, as described previously for the KZN viruses, were again evident. Notably, all of the viruses that were obtained from the EC province belonged to subfamily A, implying a closer relationship with these coastal region viruses than with the viruses of subfamily B. Even so, the EC viruses and the KZN viruses are separable within the subfamily A, suggesting a common introduction followed by a degree of independent evolution (Fig. 3). Significantly, RABV isolates from jackals and dogs (dg373.97, dg224.98, j596.99) that were obtained from provinces to the north of KZN (southeastern Mpumalanga; Fig. 4), clustered within the subfamily B collection of viruses, previously shown as viruses specific to northern KZN (bootstrap, 97%).

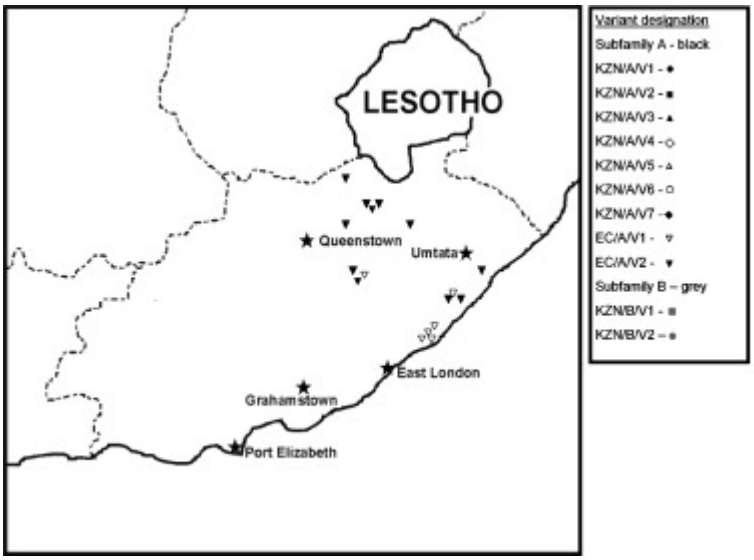

Fig. 5. A map of the Eastern Cape (EC) province demonstrating the approximate geographic origin of rabies viruses that were sequenced during the course of this study. Symbols correspond to those that were used to indicate the respective viral groupings on the phylogenetic tree in **Fig. 3**.

Table 2.

Reference rabies virus sequences included in this analysis (**Sabeta et al., 2003** and **Nel et al., 2005**)

Number	Lab reference number[a]	Species[b]	Country of isolation	Year of isolation	Locality	Coordinates (longitude–latitude)	Genbank accession number
1	dgA95.755	Canine	South Africa	1995	Amanzintoti	30°54′–30°04′	**AF303081**
2	dg373.97	Canine	South Africa	1997	Baberton	31°48′–25°42′	**AF303069**
3	dg224.98	Canine	South Africa	1998	Ermelo	29°59′–26°31′	**AF177098**
5	j596.99	*C. mesomelas*	South Africa	1999	Piet Retief	31°12′–29°33′	**AF303063**
5	d21057	Canine	Zimbabwe	1992	Muzarabani	31°12′–16°19′	**AF177064**
6	d21869	Canine	Zimbabwe	1993	Nyakasoro, Pfungwe	32°15′–16°49′	**AF177069**
7	d22547	Canine	Zimbabwe	1994	Kumutsenzere, Masoso	31°47′–16°22′	**AF177070**
8	d19385	Canine	Zimbabwe	1991	Zaka	31°34′–20°12′	**AF303080**
9	d24505	Canine	Zimbabwe	1996	Gutu	31°10′–19°37′	**AF177075**
10	d16387	Canine	Zimbabwe	1986	Zhombe	29°22′–18°41′	**AF177057**
11	d21428	Canine	Zimbabwe	1993	Chipinge	32°43′–20°22′	**AF177065**
12	j23275	*C. adustus*	Zimbabwe	1995	Bindura	31°21′–16°56′	**AF177089**
13	d24465	Canine	Zimbabwe	1996	Wendza	31°43′–18°52′	**AF177074**

Number	Lab reference number[a]	Species[b]	Country of isolation	Year of isolation	Locality	Coordinates (longitude–latitude)	Genbank accession number
14	j17711	*C. mesomelas*	Zimbabwe	1988	Turk Mine	30°49′–19°27′	**AF177087**
15	j306.98	*C. mesomelas*	South Africa	1998	Warmbaths	28°07′–24°51′	**AF177105**
16	j673.99	*C. mesomelas*	South Africa	1999	Potgietersrus	28°36′–22°43′	**AF303061**
17	j717.99	*C. mesomelas*	South Africa	1999	Pietersburg	29°29′–23°42′	**AF303064**
18	j23374	*C. mesomelas*	Zimbabwe	1995	Bulawayo	28°47′–20°15′	**AF177091**
19	j631	*C. mesomelas*	South Africa	1999	Soutpansberg	30°05′–22°03′	**AF303060**
20	m669.90	*Cynictis penicillata*	South Africa	1990	Grootgewaagd	29°52′–26°42′	**AF079907**
21	o377.99	*O. megalotis*	South Africa	1999	Gordonia	30°28′–18°09′	**AF177119**
22	o414.96	*O. megalotis*	South Africa	1996	Beaufort West	22°47′–32°22′	**AF177112**
23	o469.99	*O. megalotis*	South Africa	1999	Kimberley	24°27′–28°47′	**AF303073**
24	o491.98	*O. megalotis*	South Africa	1998	Petrusburg	25°29′–29°23′	**AF303059**
25	o491.98	*O. megalotis*	South Africa	1998	Petrusburg	25°29′–29°23′	**AF303059**
26	o578.95	*O. megalotis*	South Africa	1995	Strydenburg/Hopetown	23°46′–29°55′	**AF177113**

Number	Lab reference number[a]	Species[b]	Country of isolation	Year of isolation	Locality	Coordinates (longitude–latitude)	Genbank accession number
27	o774.95	*O. megalotis*	South Africa	1995	Carnarvon	21°56′–31°13′	**AF177115**

[a] Laboratory reference numbers are preceded by the following symbols to indicate the host species of isolation, dog (dg, d), bat eared fox (o), jackal (j).

[b] We use the term ‘canine’ to refer to domestic dogs (*C. familiaris*).

4. Discussion

All the viruses in our panel were found to belong to the canid RABV biotype. The fact that the mongoose RABV biotype was not encountered was not unexpected, as the Drakensberg Mountains, a massive range on the western and northern border regions of the KZN and EC provinces, respectively, provides an effective barrier to the natural movement of wildlife from the mongoose rabies endemic regions of the central plateau. Rabies viruses in the KZN province segregated into two major groups (subfamilies) and the distribution pattern of the two subfamilies in their respective regions of isolation suggests that they represented independent epidemic fronts. The fact that the majority of viruses from the KZN province clustered into subfamily A, led us to believe that this lineage was representative of the present day core of the epidemic. Within this subfamily, which was not monophyletic, the order of the divergence events together with geographic distribution, suggested that rabies was likely to have been disseminated via the translocation of infected dogs. For instance, the order of the divergence events and geographic distribution for cluster KZN/A/V1-KZN-A/V3 and KZN/A/V4 as elucidated in the Section 3, was in accordance with an epidemic that had spread sequentially southwards along the northern coastal regions of the province, whereas the simultaneous divergence of clusters KZN/A/7 and KZN/A/V5-KZN/A/V6 (isolated from southern KZN), suggested that the group 1 common ancestor was also introduced independently into southern KZN during the same time period. These translocation events are likely to have included the motorized transportation of infected dogs. It is noteworthy that a major transport artery, the N2 highway, runs all along the eastern seaboard of KZN. We identified a single independent cluster, comprised of three viruses, from the southern border regions of KZN. However, we could subsequently demonstrate by sequence analysis of viruses from the bordering EC province that isolates belonging to this cluster formed part of two larger rabies cycles (cluster EC/A/V1 and EC/A/V2). Both these clusters have a wide distribution throughout the EC and one of these, EC/A/V2, had evidently spread northwards into southern KZN. Importantly, the order of the divergence events suggested that the EC cycles became established at an earlier time period than the present-day cycles of subfamily A, that were identified from the coastal regions of the KZN province (KZN group of subfamily A viruses) (Fig. 3).

Viruses in the second major group (subfamily B) were isolated from the northern regions of KZN and presented as two clusters. The percentage intrinsic sequence divergence for the two clusters was very low, with many of the sequences in both groups being identical. This may suggest that these RABV cycles represent very recent introductions. Perhaps not surprisingly, this lineage was found to be related to viruses (dog and jackal) from a northerly bordering province (Mpumalanga). The importance of this observation lies in the implicated role that jackals may have in the epidemiology of the disease within northern KZN itself, and further suggested that this viral lineage may have an underestimated, broad distribution throughout the neighbouring Mpumalanga province. The northern region of the KZN province coincides with the known distribution of the black backed jackal, and boasts intensive agricultural activities such as game ranching and cattle farming (URL: http://agriculture.kzntl.gov.za.dae.index.aspx/?ID=4, [Accessed 21 April 2005]). Such activities have previously been shown to provide ideal ecological conditions for the proliferation of the said vector species, allowing them to reach a sufficient population density at which they are able to sustain virus transmission (Estes, 1992, Bingham and Foggin, 1993 and Bingham, 2005). It is clear that rabies surveillance and epidemiological study in this region of KZN should continue to investigate and monitor the involvement of this wildlife species, towards elucidating the radiation and independence and/or co-dependence of dog and jackal rabies cycles.

In an extension of our investigation, rabies viruses from the EC province of RSA were analyzed for the first time, and provided us with a better understanding of the epidemiological relationship that exists between the KZN epidemic and newly recognized rabies cycles in the EC province. It was apparent that the majority of viruses from the KZN and EC provinces belonged to a unique viral lineage (subfamily A), which was in general distinguishable from RABV isolates that were obtained from elsewhere in southern Africa. However, beyond the collateral distinction of the subfamily A collection of viruses, they were found to segregate into three distinct groups that were unique to either the KZN (KZN group of subfamily A viruses) or the EC (EC/A/V1, EC/A/V2) province. Cumulatively our findings suggested that the most recent common ancestor of this subfamily could have been introduced on more than one occasion into either of the

KZN and EC provinces. This observation is not entirely consistent with suggestions that the outbreaks in KZN and the EC provinces were commonly introduced through the sequential southwards spread of the disease along the eastern coastal belt of South Africa (Bishop et al., 2003 and Swanepoel, 2005).

We believe that there is one likely explanation for the phylogenetic and regional divisions within the subfamily A lineage of KZN and EC viruses. Consider that two epidemics had occurred among dogs in the affected provinces in recent times. The first epidemic spread through the KZN province from 1964 to 1968 and apparently did not enter the EC province (Bishop et al., 2003 and Swanepoel, 2005), whereas the second epidemic, which started in 1976, is known to have spread as far southwards as the EC province, from where it was reported since 1987 (Bishop et al., 2003 and Swanepoel, 2005). We suggest that during the 1964–1968 epidemic, rabies did in fact spread as far southwards as the northern regions of the EC province, which it entered to initiate outbreaks among local populations of domestic dogs. Although the first epidemic in KZN province was brought under control, the focus in the EC province probably persisted in the locations bordering on KZN, which was a former apartheid homeland, the Transkei. Here, rabies surveillance would have been nonexistent throughout the intervening time period between the first and second epidemic, i.e. from the late 1960's to 80's. The persistence of the initial outbreak in the EC province (Transkei), and the reintroduction of the disease into the coastal regions of KZN during the second epidemic, could then account for the phylogenetic patterns that emerged in our analyses. Finally, the delineation of the geographical distribution of the respective EC and KZN sequence clusters in this analysis, allows us to comment on the bidirectional movement of variants across the border between the two provinces. For example, viruses belonging to the KwaZulu Natal group of subfamily A viruses were isolated from the EC, whereas an isolate belonging to cluster EC/A/V2 (primarily composed of viruses from EC), was also isolated from KZN. It is probable that the movement of these variants have been facilitated by the transport of infected dogs along the major N2 highway, which spans the border regions between the two provinces, highlighting the requirement for stricter control on animal movement along this stretch of highway.

This study is one of the most comprehensive and detailed for any specific epidemic of dog rabies in Africa to date. Most importantly, molecular epidemiology allowed us to track and describe the emergence and continued expansion of rabies in a defined region of southern Africa. We were also able to investigate historical dogma, to highlight the potential future involvement of wildlife in the epidemic, and to generate a sequence database for future surveillance efforts. It is our contention that the factors driving the proliferation of rabies in South Africa also apply throughout the rest of southern Africa and the larger continent as a whole. As the toll of rabies in Africa continues to escalate, the need for a refined approach and a serious focus on rabies, which so greatly benefited Europe and the Americas, should not be negated.

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☆The Genbank accession numbers for the sequences reported in this paper are DQ841403-DQ841549.

☆☆We present the first molecular epidemiological evidence for the emerging distribution patterns of rabies in the coastal region of southern Africa, and resolve an epidemiological scenario that may well apply to much of the African continent.

Corresponding author. Tel.: +27 12 420 3622; fax: +27 12 420 3266.