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Genetic Tracking of the Raccoon Variant of Rabies Virus in Eastern North America

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ABSTRACT

To gain insight into the incursion of the raccoon variant of rabies into the raccoon population in three Canadian provinces, a collection of 192 isolates of the raccoon rabies virus (RRV) strain was acquired from across its North American range and was genetically characterized. A 516-nucleotide segment of the non-coding region between the G and L protein open reading frames, corresponding to the most variable region of the rabies virus genome, was sequenced. This analysis identified 119 different sequences, and phylogenetic analysis of the dataset supports the documented history of RRV spread. Three distinct geographically restricted RRV lineages were identified. Lineage 1 was found in Florida, Alabama and Georgia and appears to form the ancestral lineage of the raccoon variant of rabies. Lineage 2, represented by just two isolates, was found only in Florida, while the third lineage appears broadly distributed throughout the rest of the eastern United States and eastern Canada. In New York State, two distinct spatially segregated variants were identified; the one occupying the western and northern portions of the state was responsible for an incursion of raccoon rabies into the Canadian province of Ontario. Isolates from New Brunswick and Quebec form distinct, separate clusters, consistent with their independent origins from neighboring areas of the United States. The data are consistent with localized northward incursion into these three separate areas with no evidence of east–west viral movement between the three Canadian provinces.

Introduction

Rabies, one of the most important worldwide zoonoses, is a viral infectious disease of the central nervous system that almost invariably causes death once clinical symptoms are evident (Jackson, 2007). The disease is responsible for over 50,000 human deaths annually, mainly in countries of Africa and Asia (World Health Organization, 2005). In North America, where human deaths due to rabies are rare, the virus however remains a significant public health issue due to its persistence in a number of wildlife reservoirs. Several distinct rabies virus strains or variants persist, each associated with a specific reservoir host, with changes in the phylogeographic distribution of each strain over time (Hanlon et al., 2007). Over the last 40 years, however, the raccoon rabies strain has been, by far, the most widely dispersed (see Biek et al., 2007).

The first record of a rabid raccoon (*Procyon lotor*) in Florida was reported in 1947, and by the mid-1950s it was apparent that a significant epizootic was in progress in the state (Burridge et al., 1986; McLean, 1971). This epizootic remained localized for over two decades until a second epizootic, since designated as the mid-Atlantic raccoon rabies outbreak (Jenkins and Winkler, 1987), was reported in West Virginia in 1977 and in Virginia the following year (Winkler and Jenkins, 1991). This new epizootic was probably the result of translocation of infected raccoons from Florida; Virginia wildlife records document the translocation of thousands of raccoons from Florida to Virginia in the late 1970s for hunting purposes (Nettles et al., 1979). This epizootic subsequently moved southwards and very rapidly northwards until all states along the eastern seaboard of the United States (US) were affected (Winkler and Jenkins, 1991; Wandeler et al., 2000). The rate of viral spread ranged from 9.5 km/year to 38.4 km/year (Biek et al., 2007). Several natural barriers slowed or prevented further movement either westwards (Appalachian mountain range) or northwards into Canada (the Great Lakes and St. Lawrence Seaway). However, despite proactive efforts to prevent further northwards expansion into Ontario by immunization of raccoon populations against rabies in areas bordering New York State (Rosatte et al., 1992, 1997; Rosatte, 2000), an incursion of raccoon rabies was reported in Ontario in July 1999 (Wandeler and Salsberg, 1999). Raccoon rabies control tactics were implemented in Ontario, and the last reported case was in 2005 (Rosatte et al., 2001, 2006, 2008, 2009a,b). Additional incursions have occurred in New Brunswick (MacInnes, 2000), between 2000 and 2002 and in Quebec, between 2006 and 2009 (see http://www.rageduratonlaveur.gouv.qc.ca/presse.asp). In each case control strategies based on those employed in Ontario were successful in eradicating the outbreak. In all outbreaks the raccoon was the principal host with occasional

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spillover into other species, particularly the striped skunk (*Mephitis mephitis*). Indeed in New Brunswick, a high proportion of the initial recorded cases of raccoon variant rabies occurred in the skunk (Nadin-Davis et al., 2006).

Rabies virus, a member of the Rhabdovirus family and *Lyssavirus* genus, is a bullet-shaped, enveloped negative strand RNA virus with an unsegmented genome of about 12 kb (reviewed in Wunner, 2007). The genome encodes for 5 viral proteins: nucleoprotein (N), phosphoprotein (P), glycoprotein (G), matrix protein (M) and polymerase or large protein (L). A relatively long non-coding region between the G and L protein open reading frames (ORFs) comprises the 3′-terminal non-coding G gene region, the G–L intergenic region and the L mRNA transcription initiation signal. In the raccoon rabies virus (RRV), these regions are 464 nucleotides (nt), 24 nt and 27 nt in length respectively, thereby yielding a total length of 515 nt (Szanto et al., 2008). The non-coding G–L region that is presumably not subject to the same mutational constraints that operate on viral coding regions is the most variable region of the lyssavirus genome (Sacramento et al., 1992; Le Mercier et al., 1997). This region has been employed as a useful target to dissect the recent evolutionary history of closely related rabies viruses in Europe (Sacramento et al., 1992), South America (Hughes et al., 2004; Sato et al., 2004), Africa (Nel et al., 2005; Sabeta et al., 2003; von Teichman et al., 1995) and Asia (Hyun et al., 2005; Meng et al., 2007) and was thus considered the most suitable target for the detailed genetic tracking required for this study.

The objectives of this study were to genetically characterize the non-coding G–L region of RRV isolates recovered from throughout the strain's North American range and to use their phylogenetic relationships to trace outbreaks to their original source. The use of samples recovered over a 24 year period (1982–2006) permitted application of Bayesian methods of analysis to this molecular dataset to estimate the date of emergence of this rabies virus strain and several of its variants. Understanding raccoon rabies epidemiology by monitoring viral spread and discriminating between local incursions and long-distance translocation events is crucial to control efforts and the ultimate eradication of this disease in North America.

Materials and methods

Virus isolates and RNA isolation

Raccoon strain rabies samples selected for this study, comprised specimens of original infected host brain tissues. Specimens were generously provided by laboratories that maintain extensive rabies virus collections including the Canadian Food Inspection Agency (CFIA), the Centers for Disease Control and Prevention (CDC) and the New York State Department of Health. As far as sample availability is allowed, we sought to include viruses representative of the spatial and temporal range exhibited by the RRV strain but with a particular emphasis on samples recovered from NY and ON in order to examine spread throughout this region in some detail. A complete listing of all specimens is given in Table 1.

Total RNA was extracted using TRIzol according to the supplier's instructions (Invitrogen, Burlington, Canada), and the RNA precipitates were resuspended in 50 μl of nuclease-free distilled water. Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland DE).

Strain identification

Samples from CFIA have been previously identified as a raccoon strain either by antigenic analysis (Nadin-Davis et al., 2001) and/or by P gene sequencing (Nadin-Davis et al., 2006); antigenic or genetic typing methods were also employed by CDC. To confirm the nature of the virus strain in all New York samples, they were subjected to a strain-specific PCR as outlined previously (Nadin-Davis et al., 1996).

cDNA synthesis, amplification and sequencing

The primers used for cDNA synthesis and polymerase chain reaction (PCR) were designed based on a complete genomic sequence of the RRV strain described previously (Szanto et al., 2008). Synthesis of cDNA was performed with 2 μg of total RNA as outlined by Nadin-Davis (1998) using 1 μM positive-sense primer Gseq-RAC1 (5′-CTCGG ATGAGCTTGAGCATCTTGT-3′) corresponding to bases 4164–4187 of RRV (GenBank accession number EU311738). The PCR was performed on a PTC-100 Programmable Thermal Controller (MJ Research Inc.) in a 25 μl reaction mixture. A 2.5 μl aliquot of cDNA was used as template for PCR using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) with 0.25 μM of negative-sense primer LysGRev-1 (5′-TCTTCTA(GT)CAAAGGAGAGTTGAG (AG)TTGTAGT-3′) that targets bases 5542–5512 of the RRV strain L gene sequence. Thermocycling profiles involved an initial denaturation for 2 min at 93 °C, followed by 35 cycles of denaturing, annealing and elongation steps (93 °C for 10 s, 48 °C for 30 s, and 68 °C for 4 min) and a final extension at 68 °C for 5 min. Amplification of fragments of the expected 1379-bp size was confirmed visually by ethidium bromide staining of 1.5% agarose gels after standard DNA electrophoresis (Sambrook et al., 1989). When amplicon yield was low, a second round of amplification was performed by a hemi-nested PCR using 0.25 μM of positive sense primer RRV-5 F (5′-TTCATCTCCCAGATGTTCAC AAA-3′), corresponding to bases 4625–4647 of the RRV strain G gene sequence, and 0.25 μM of primer LysGRev-1 under the same thermocycling conditions as used for the first round of PCR. Amplicons were purified using ExoSAP-IT (USB), following the manufacturer's instructions, and then sequenced with both forward (RRV-5 F) and reverse (LysGRev-1) primers on a MegaBase 1000 (GE Healthcare) 96 well plate DNA sequencer using the DYEnamic™ ET terminator cycle sequencing kit (GE Healthcare). In some instances, additional sequencing was performed with the following internal primers: RRV-8 F (5′-GCTGATGATTGCAGGTG CTCTA-3′), corresponding to bases 4695–4716 of positive-sense G gene sequence of RRV, and RRV-8 R (5′-CTCTCAGACTCGACTGG ATCGA-3′), corresponding to negative-sense sequence of bases 5443–5464 of the RRV L gene. For all isolates used in the study, nucleotide sequence was determined for both strands of the amplicon over a range corresponding to bases 4891–5405 of the RRV genome (GenBank accession EU311738).

Phylogenetic analysis

The sequences were manually edited and compiled in BioEdit (Hall, 1999). Alignments were performed using ClustalW (Thompson et al., 1994) in BioEdit. Phylogenetic analysis using the neighbor joining (NJ) method with Kimura (1980) evolutionary distance correction statistics was performed using the MEGA 4 program (Tamura et al., 2007). The branching pattern of the resulting tree was statistically evaluated by bootstrap analysis of 1000 data replicates (Felsenstein, 1985; Dopazo, 1994) with values of 70% or greater considered significant (Hillis and Bull, 1993).

Bayesian analysis

Modeltest (v.3.7), available from http://www.darwin.uvigo.es/software/modeltest.html (Posada and Crandall, 1998), was applied to the complete sequence database to identify the GTR + G (General Time Reversible with rate variation among sites) nucleotide substitution model as giving the best fit to the data. Using this model, the dataset was analyzed by the BEAST package (v.1.4.8). This software implements a Bayesian coalescent approach, using Markov-Chain Monte Carlo (MCMC) sampling to infer time-measured phylogenies using molecular sequences (Drummond and Rambaut, 2007). The dataset was analyzed by multiple runs, which compared use of strict vs. relaxed molecular clocks and constant vs. exponential population

Table 1
Raccoon rabies isolates employed in this study.

Accession no.	Isolate name	Species	Date[a]	Country	Prov./State	County
GQ851532	RRV ON-99-2	Raccoon	26-Jul-99	Canada	Ontario	Leeds
GQ851533	RRV ON-99-3	Raccoon	17-Sep-99	Canada	Ontario	Grenville
GQ851534	RRV ON-99-4	Raccoon	10-Dec-99	Canada	Ontario	Frontenac
GQ851535	RRV ON-99-5	Raccoon	16-Dec-99	Canada	Ontario	Leeds
GQ851536	RRV ON-99-6	Raccoon	29-Dec-99	Canada	Ontario	Grenville
GQ851537	RRV ON-00-14	Raccoon	25-Jan-00	Canada	Ontario	Leeds
GQ851538	RRV ON-00-15	Raccoon	1-Feb-00	Canada	Ontario	Leeds
GQ851539	RRV ON-00-17	Raccoon	14-Feb-00	Canada	Ontario	Grenville
GQ851540	RRV ON-00-22	Raccoon	7-Mar-00	Canada	Ontario	Leeds
GQ851541	RRV ON-00-24	Raccoon	7-Apr-00	Canada	Ontario	Grenville
GQ851542	RRV ON-00-30	Raccoon	17-May-00	Canada	Ontario	Grenville
GQ851543	RRV ON-00-32	Raccoon	5-Jun-00	Canada	Ontario	Leeds
GQ851544	RRV ON-00-34	Raccoon	12-Jun-00	Canada	Ontario	Leeds
GQ851545	RRV ON-00-38	Raccoon	23-Aug-00	Canada	Ontario	Leeds
GQ851546	RRV ON-00-42	Raccoon	15-Sep-00	Canada	Ontario	Leeds
GQ851547	RRV ON-00-44	Raccoon	5-Oct-00	Canada	Ontario	Leeds
GQ851548	RRV ON-01-66	Raccoon	8-Feb-01	Canada	Ontario	Leeds
GQ851549	RRV ON-01-70	Raccoon	20-Mar-01	Canada	Ontario	Leeds
GQ851550	RRV ON-01-110	Raccoon	4-May-01	Canada	Ontario	Leeds
GQ851551	RRV ON-01-128	Raccoon	3-Jul-01	Canada	Ontario	Leeds
GQ851552	RRV ON-01-129	Raccoon	4-Jul-01	Canada	Ontario	Leeds
GQ851553	RRV ON-01-131	Raccoon	6-Jul-01	Canada	Ontario	Leeds
GQ851554	RRV ON-01-138	Raccoon	8-Aug-01	Canada	Ontario	Leeds
GQ851555	RRV ON-01-139	Raccoon	16-Aug-01	Canada	Ontario	Leeds
GQ851556	RRV ON-01-143	Raccoon	24-Aug-01	Canada	Ontario	Leeds
GQ851557	RRV ON-01-150	Raccoon	13-Dec-01	Canada	Ontario	Leeds
GQ851558	RRV ON-01-152	Raccoon	24-Dec-01	Canada	Ontario	Leeds
GQ851559	RRV ON-02-155	Raccoon	18-Jan-02	Canada	Ontario	Leeds
GQ851560	RRV ON-02-160	Raccoon	19-Mar-02	Canada	Ontario	Leeds
GQ851561	RRV ON-02-162	Raccoon	29-May-02	Canada	Ontario	Leeds
GQ851562	RRV ON-02-164	Raccoon	19-Jun-02	Canada	Ontario	Leeds
GQ851563	RRV ON-02-165	Raccoon	21-Jun-02	Canada	Ontario	Leeds
GQ851564	RRV ON-02-167	Raccoon	2-Oct-02	Canada	Ontario	Grenville
GQ851565	RRV ON-02-170	Raccoon	24-Oct-02	Canada	Ontario	Grenville
GQ851566	RRV ON-02-172	Raccoon	29-Oct-02	Canada	Ontario	Grenville
GQ851567	RRV ON-03-180	Raccoon	19-Mar-03	Canada	Ontario	Grenville
GQ851568	RRV ON-03-183	Raccoon	14-Apr-03	Canada	Ontario	Grenville
GQ851569	RRV ON-03-185	Raccoon	17-Apr-03	Canada	Ontario	Leeds
GQ851570	RRV ON-03-187	Raccoon	5-May-03	Canada	Ontario	Leeds
GQ851571	RRV ON-03-188	Raccoon	12-May-03	Canada	Ontario	Leeds
GQ851572	RRV ON-03-190	Raccoon	10-Jun-03	Canada	Ontario	Grenville
GQ851573	RRV ON-04-192	Raccoon	16-Aug-04	Canada	Ontario	Grenville
GQ851574	RRV NB-00-48	Skunk	31-Oct-00	Canada	New Brunswick	Charlotte
GQ851575	RRV NB-00-51	Raccoon	16-Nov-00	Canada	New Brunswick	Charlotte
GQ851576	RRV NB-00-52	Raccoon	16-Nov-00	Canada	New Brunswick	Charlotte
GQ851577	RRV NB-00-54	Raccoon	16-Nov-00	Canada	New Brunswick	Charlotte
GQ851578	RRV NB-01-59	Raccoon	2-Jan-01	Canada	New Brunswick	Charlotte
GQ851579	RRV NB-01-60	Skunk	2-Jan-01	Canada	New Brunswick	Charlotte
GQ851580	RRV NB-01-62	Raccoon	18-Jan-01	Canada	New Brunswick	Charlotte
GQ851581	RRV NB-01-67	Raccoon	20-Feb-01	Canada	New Brunswick	Charlotte
GQ851582	RRV NB-01-69	Raccoon	20-Mar-01	Canada	New Brunswick	Charlotte
GQ851583	RRV NB-01-77	Raccoon	30-Mar-01	Canada	New Brunswick	Charlotte
GQ851584	RRV NB-01-82	Raccoon	4-Apr-01	Canada	New Brunswick	Charlotte
GQ851585	RRV NB-01-84	Raccoon	11-Apr-01	Canada	New Brunswick	Charlotte
GQ851586	RRV NB-01-88	Raccoon	12-Apr-01	Canada	New Brunswick	Charlotte
GQ851587	RRV NB-01-90	Raccoon	12-Apr-01	Canada	New Brunswick	Charlotte
GQ851588	RRV NB-01-92	Raccoon	18-Apr-01	Canada	New Brunswick	Charlotte
GQ851589	RRV NB-01-103	Raccoon	26-Apr-01	Canada	New Brunswick	Charlotte
GQ851590	RRV NB-01-106	Raccoon	2-May-01	Canada	New Brunswick	Charlotte
GQ851591	RRV NB-01-107	Raccoon	2-May-01	Canada	New Brunswick	Charlotte
GQ851592	RRV NB-01-116	Raccoon	24-May-01	Canada	New Brunswick	Charlotte
GQ851593	RRV NB-01-118	Raccoon	31-May-01	Canada	New Brunswick	Charlotte
GQ851594	RRV NB-01-124	Raccoon	21-Jun-01	Canada	New Brunswick	Charlotte
GQ851595	RRV NB-01-140	Raccoon	16-Aug-01	Canada	New Brunswick	Charlotte
GQ851596	RRV NB-01-145	Raccoon	18-Sep-01	Canada	New Brunswick	Charlotte
GQ851597	RRV NB-01-147	Raccoon	31-Oct-01	Canada	New Brunswick	Charlotte
GQ851598	RRV NB-02-158	Raccoon	30-Jan-02	Canada	New Brunswick	Charlotte
GQ851599	RRV NB-02-163	Raccoon	30-May-02	Canada	New Brunswick	Charlotte
GQ851600	RRV QC-06-197	Raccoon	2-Jun-06	Canada	Quebec	Brome-Missisquoi
GQ851601	RRV QC-06-199	Raccoon	11-Sep-06	Canada	Quebec	Les Jard. Nepierville
GQ851502	RRV NY-90-280	Raccoon	4-May-90	U.S.A.	New York	Steuben
GQ851503	RRV NY-90-281	Raccoon	3-Jul-90	U.S.A.	New York	Sullivan
GQ851504	RRV NY-90-282	Raccoon	10-Jul-90	U.S.A.	New York	Sullivan
GQ851505	RRV NY-90-283	Raccoon	10-Jul-90	U.S.A.	New York	Sullivan
GQ851506	RRV NY-90-284	Raccoon	17-Jul-90	U.S.A.	New York	Other

Table 1 (*continued*)

Accession no.	Isolate name	Species	Date[a]	Country	Prov./State	County
GQ851507	RRV NY-90-285	Raccoon	17-Jul-90	U.S.A.	New York	Sullivan
GQ851508	RRV NY-90-287	Raccoon	19-Jul-90	U.S.A.	New York	Steuben
GQ851509	RRV NY-90-288	Raccoon	7-Aug-90	U.S.A.	New York	Sullivan
GQ851510	RRV NY-90-289	Raccoon	7-Aug-90	U.S.A.	New York	Sullivan
GQ851511	RRV NY-90-290	Raccoon	8-Aug-90	U.S.A.	New York	Sullivan
GQ851512	RRV NY-90-291	Raccoon	15-Aug-90	U.S.A.	New York	Chemung
GQ851513	RRV NY-90-292	Raccoon	30-Aug-90	U.S.A.	New York	Orange
GQ851514	RRV NY-90-293	Raccoon	18-Sep-90	U.S.A.	New York	Cattaraugus
GQ851515	RRV NY-90-294	Raccoon	23-Oct-90	U.S.A.	New York	Chemung
GQ851516	RRV NY-90-295	Raccoon	26-Oct-90	U.S.A.	New York	Allegany
GQ851517	RRV NY-90-296	Raccoon	16-Nov-90	U.S.A.	New York	Allegany
GQ851518	RRV NY-90-297	Raccoon	6-Nov-90	U.S.A.	New York	Chemung
GQ851519	RRV NY-90-298	Raccoon	20-Nov-90	U.S.A.	New York	Cattaraugus
GQ851520	RRV NY-90-299	Raccoon	28-Nov-90	U.S.A.	New York	Orange
GQ851521	RRV NY-90-300	Raccoon	17-Dec-90	U.S.A.	New York	Steuben
GQ851522	RRV NY-90-301	Raccoon	18-Dec-90	U.S.A.	New York	Cattaraugus
GQ851523	RRV NY-91-303	Raccoon	21-Feb-91	U.S.A.	New York	Delaware
GQ851524	RRV NY-91-304	Raccoon	21-Feb-91	U.S.A.	New York	Broome
GQ851525	RRV NY-91-305	Raccoon	5-Apr-91	U.S.A.	New York	Westchester
GQ851526	RRV NY-93-308	Raccoon	23-Mar-93	U.S.A.	New York	Yates
GQ851527	RRV NY-93-309	Raccoon	12-Nov-93	U.S.A.	New York	Cayuga
GQ851528	RRV NY-93-310	Raccoon	2-Dec-93	U.S.A.	New York	Ontario
GQ851529	RRV NY-95-311	Raccoon	27-Jan-95	U.S.A.	New York	Saratoga
GQ851530	RRV NY-95-312	Raccoon	12-Jul-95	U.S.A.	New York	Clinton
GQ851531	RRV NY-95-313	Raccoon	22-Dec-95	U.S.A.	New York	Jefferson
GQ851454	RRV NY-03-1	Raccoon	14-May-03	U.S.A.	New York	Westchester
GQ851455	RRV NY-03-2	Raccoon	16-May-03	U.S.A.	New York	Livingston
GQ851456	RRV NY-03-3	Raccoon	16-May-03	U.S.A.	New York	Cayuga
GQ851457	RRV NY-03-4	Skunk	16-May-03	U.S.A.	New York	Ulster
GQ851458	RRV NY-03-5	Raccoon	19-May-03	U.S.A.	New York	Otsego
GQ851459	RRV NY-03-6	Raccoon	20-May-03	U.S.A.	New York	Wayne
GQ851460	RRV NY-03-7	Raccoon	21-May-03	U.S.A.	New York	Cayuga
GQ851461	RRV NY-03-9	Raccoon	22-May-03	U.S.A.	New York	Rensselaer
GQ851462	RRV NY-03-22	Skunk	9-Jun-03	U.S.A.	New York	Cortland
GQ851463	RRV NY-03-29	Skunk	20-Jun-03	U.S.A.	New York	Oswego
GQ851464	RRV NY-03-30	Raccoon	23-Jun-03	U.S.A.	New York	Monroe
GQ851465	RRV NY-03-34	Skunk	30-Jun-03	U.S.A.	New York	Montgomery
GQ851466	RRV NY-03-36	Raccoon	1-Jul-03	U.S.A.	New York	Genesee
GQ851467	RRV NY-03-37	Skunk	1-Jul-03	U.S.A.	New York	Other
GQ851468	RRV NY-03-39	Raccoon	7-Jul-03	U.S.A.	New York	Rensselaer
GQ851469	RRV NY-03-40	Raccoon	8-Jul-03	U.S.A.	New York	Genesee
GQ851470	RRV NY-03-41	Bobcat	9-Jul-03	U.S.A.	New York	Orange
GQ851471	RRV NY-03-42	Raccoon	9-Jul-03	U.S.A.	New York	Cayuga
GQ851472	RRV NY-03-43	Raccoon	10-Jul-03	U.S.A.	New York	Seneca
GQ851473	RRV NY-03-45	Raccoon	17-Jul-03	U.S.A.	New York	Steuben
GQ851474	RRV NY-03-46	Fox, gray	17-Jul-03	U.S.A.	New York	Tioga
GQ851475	RRV NY-03-47	Skunk	18-Jul-03	U.S.A.	New York	Rockland
GQ851476	RRV NY-03-51	Raccoon	25-Jul-03	U.S.A.	New York	Montgomery
GQ851477	RRV NY-03-52	Raccoon	28-Jul-03	U.S.A.	New York	Albany
GQ851478	RRV NY-03-60	Raccoon	19-Aug-03	U.S.A.	New York	Cayuga
GQ851479	RRV NY-03-62	Raccoon	20-Aug-03	U.S.A.	New York	Westchester
GQ851480	RRV NY-03-70	Raccoon	29-Aug-03	U.S.A.	New York	Cattaraugus
GQ851481	RRV NY-03-75	Raccoon	4-Sep-03	U.S.A.	New York	Cayuga
GQ851482	RRV NY-03-76	Raccoon	4-Sep-03	U.S.A.	New York	Albany
GQ851483	RRV NY-03-77	Raccoon	8-Sep-03	U.S.A.	New York	Orleans
GQ851484	RRV NY-03-78	Raccoon	10-Sep-03	U.S.A.	New York	Genesee
GQ851485	RRV NY-03-83	Raccoon	22-Sep-03	U.S.A.	New York	Westchester
GQ851486	RRV NY-03-92	Raccoon	30-Sep-03	U.S.A.	New York	Madison
GQ851487	RRV NY-03-96	Raccoon	6-Oct-03	U.S.A.	New York	Rockland
GQ851488	RRV NY-03-97	Skunk	7-Oct-03	U.S.A.	New York	Madison
GQ851489	RRV NY-03-98	Raccoon	8-Oct-03	U.S.A.	New York	Tioga
GQ851490	RRV NY-03-99	Fox, gray	9-Oct-03	U.S.A.	New York	Albany
GQ851491	RRV NY-03-100	Raccoon	15-Oct-03	U.S.A.	New York	Westchester
GQ851492	RRV NY-03-113	Raccoon	4-Nov-03	U.S.A.	New York	Rensselaer
GQ851493	RRV NY-03-115	Raccoon	4-Nov-03	U.S.A.	New York	Schuyler
GQ851494	RRV NY-03-116	Raccoon	5-Nov-03	U.S.A.	New York	Steuben
GQ851495	RRV NY-03-124	Raccoon	19-Nov-03	U.S.A.	New York	Rensselaer
GQ851496	RRV NY-03-138	Skunk	3-Dec-03	U.S.A.	New York	Saratoga
GQ851497	RRV NY-03-141	Raccoon	11-Dec-03	U.S.A.	New York	Columbia
GQ851498	RRV NY-03-146	Raccoon	19-Dec-03	U.S.A.	New York	Dryden
GQ851499	RRV NY-03-152	Fox, red	24-Dec-03	U.S.A.	New York	Saratoga
GQ851500	RRV NY-03-154	Raccoon	25-Dec-03	U.S.A.	New York	Orleans
GQ851501	RRV NY-03-156	Raccoon	30-Dec-03	U.S.A.	New York	Westchester
GQ851432	RRV AL-98-166	Raccoon	8-Jan-98	U.S.A.	Alabama	
GQ851433	RRV AL-98-167	Raccoon	8-Jan-98	U.S.A.	Alabama	
GQ851434	RRV AL-98-168	Raccoon	12-Jan-98	U.S.A.	Alabama	

(*continued on next page*)

Table 1 (*continued*)

Accession no.	Isolate name	Species	Date[a]	Country	Prov./State	County
GQ851435	RRV AL-98-169	Raccoon	8-Jan-98	U.S.A.	Alabama	
GQ851436	RRV AL-98-170	Raccoon	10-Jan-98	U.S.A.	Alabama	
GQ851437	RRV GA-83-174	Raccoon	1-Jan-83	U.S.A.	Georgia	
GQ851438	RRV GA-83-175	Raccoon	1-Jan-83	U.S.A.	Georgia	
GQ851439	RRV GA-83-177	Raccoon	1-Jan-83	U.S.A.	Georgia	
GQ851440	RRV GA-03-178	Raccoon	22-Sep-03	U.S.A.	Georgia	
GQ851441	RRV VA-93-179	Raccoon	1-Mar-93	U.S.A.	Virginia	
GQ851442	RRV VA-92-183	Raccoon	1-Jul-92	U.S.A.	Virginia	
GQ851443	RRV VA-92-185	Raccoon	1-Jun-92	U.S.A.	Virginia	
GQ851444	RRV VA-84-186	Raccoon	1-Feb-84	U.S.A.	Virginia	
GQ851445	RRV VA-82-188	Raccoon	1-Oct-82	U.S.A.	Virginia	
GQ851446	RRV WV-87-190	Raccoon	1-Aug-87	U.S.A.	West Virginia	
GQ851447	RRV WV-93-191	Raccoon	1-Mar-93	U.S.A.	West Virginia	
GQ851448	RRV MD-82-195	Raccoon	1-Apr-82	U.S.A.	Maryland	
GQ851449	RRV MD-82-196	Raccoon	1-Sep-82	U.S.A.	Maryland	
GQ851451	RRV NH-92-198	Raccoon	1-Apr-92	U.S.A.	New Hampshire	
GQ851452	RRV NH-92-199	Raccoon	1-Dec-92	U.S.A.	New Hampshire	
GQ851450	RRV NJ-01-201	Pony	1-Nov-01	U.S.A.	New Jersey	
GQ851453	RRV PA-02-202	Cat	8-May-02	U.S.A.	Pennsylvania	
GQ851425	RRV FL-88-205	Raccoon	19-Jan-88	U.S.A.	Florida	
GQ851412	RRV FL-87-207	Raccoon	19-Oct-87	U.S.A.	Florida	
GQ851413	RRV FL-87-209	Raccoon	7-Oct-87	U.S.A.	Florida	
GQ851426	RRV FL-88-214	Raccoon	12-Jan-88	U.S.A.	Florida	
GQ851414	RRV FL-87-215	Raccoon	1-Jan-87	U.S.A.	Florida	
GQ851415	RRV FL-87-217	Raccoon	1-May-87	U.S.A.	Florida	
GQ851416	RRV FL-87-219	Raccoon	1-Mar-87	U.S.A.	Florida	
GQ851417	RRV FL-87-221	Raccoon	17-Mar-87	U.S.A.	Florida	
GQ851418	RRV FL-87-228	Raccoon	1-Jan-87	U.S.A.	Florida	
GQ851427	RRV FL-88-233	Raccoon	1-Jan-88	U.S.A.	Florida	
GQ851428	RRV FL-88-234	Raccoon	1-Dec-88	U.S.A.	Florida	
GQ851419	RRV FL-87-239	Raccoon	1-Dec-87	U.S.A.	Florida	
GQ851420	RRV FL-87-244	Raccoon	1-Nov-87	U.S.A.	Florida	
GQ851429	RRV FL-88-248	Raccoon	1-Jan-88	U.S.A.	Florida	
GQ851421	RRV FL-87-249	Raccoon	1-Apr-87	U.S.A.	Florida	
GQ851422	RRV FL-87-258	Raccoon	1-Jun-87	U.S.A.	Florida	
GQ851423	RRV FL-87-262	Raccoon	1-Jan-87	U.S.A.	Florida	
GQ851410	RRV FL-86-264	Raccoon	1-Nov-86	U.S.A.	Florida	
GQ851431	RRV FL-99-266	Raccoon	1-Jan-99	U.S.A.	Florida	
GQ851430	RRV FL-88-269	Raccoon	1-Jan-88	U.S.A.	Florida	
GQ851424	RRV FL-87-270	Raccoon	1-Nov-87	U.S.A.	Florida	
GQ851411	RRV FL-86-274	Raccoon	1-Nov-86	U.S.A.	Florida	

[a] Date of sample receipt at diagnostic laboratory.

growth. Optimal parameters were identified based on the likelihood values obtained for each run. The optimal analysis, which invoked a relaxed log-normal molecular clock and exponential growth, employed 20 million steps with discard of the first 2 million steps as a burn-in period followed by sampling every 2000 steps. Effective sampling sizes (ESSs) > 100 were obtained for all parameters with the majority having ESSs > 200, and analysis was performed twice to ensure convergence as indicated by the very close likelihood values (−3293.9 and −3294.9) obtained. Output was viewed in Tracer (v.1.4.8), and employed by TreeAnnotator (v.1.4.8) to generate a maximum clade credibility (MCC) tree with final graphical output generated by the Figtree software (v.1.1.2); all software is readily available through links on the BEAST web page (http://www.beast.bio.ed.ac.uk/).

Map generation

Map was generated using ArcGIS v.9.2 (Environmental Systems Research Institute Inc., Redlands, CA, USA).

Results

Nucleotide sequence comparison of the G–L region

An alignment of all 192 sequences identified 119 distinct variants each exhibiting at least one different base substitution along the length of the targeted G–L sequence. In addition a single base insertion was identified for certain Ontario and New York isolates such that the transcription termination and polyadenylation (TTP) signal was modified from the normal $WG(A)_7$ motif to $WG(A)_8$ thereby resulting in an alignment of 516 bases. Overall a surprisingly high level of sequence conservation was observed, with the most distantly related Florida and Ontario samples having a nucleotide sequence identity of approximately 95%. Of the 516 nucleotides examined, 293 (56.8%) were absolutely conserved among all 192 RRV isolates. Interestingly, nucleotide substitutions were not randomly distributed across this region as might be expected for a region of limited function, but four relatively conserved stretches of sequence were identified: between positions 89–111, 194–237, 327–394 and 448–502 (Fig. 1).

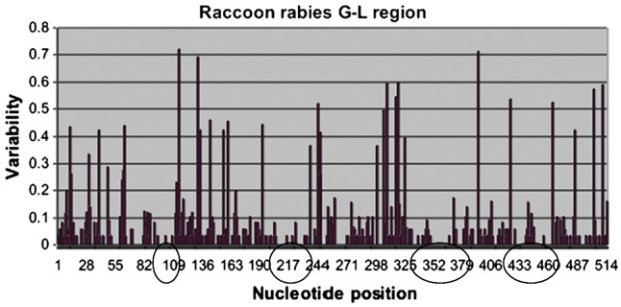


Fig. 1. Nucleotide sequence variability of the raccoon rabies G–L non-coding region over a 516 bp alignment of 192 isolates. The relatively conserved regions are encircled.

Table 2
Nucleotide substitution observed in isolates according to their geographical origin.

NT #	ON	NY	NB	MD	NH	NJ	PQ	PA	AL	GA	FL
64	A	A	G	A	A[a]	A	A	A	A	A[b]	A
144	T	T	T	T	T	T	T	T	C	C	C
309	G	A	G	A	A	A	A	G	G	G	G
317	G	A[c]	A	A	A	A	A	A	A	A	A[d]
320	T	C	T	T	C	C	C	T	T	T	C
388	C	C	C	C	C	C	T	C	C	C	C
395	T	T/C	C	C	C	C	C	C	C	C	C
465	A[e]	–[f]	–	–	–	–	–	–	–	–	–
503	C	T	C	C	T	T	T	T	C	C	C
512	T	T/G	G	G	G	G	G	G	G	G	G

[a] G for one NH isolate.
[b] T for one GA isolate.
[c] G for 2 NY isolates.
[d] G for one FL isolate.
[e] Except for two ON isolates 99-2 and 99-4 the latter of which was from Wolfe Island and hence part of a separate incursion.
[f] Except for two isolates, NY-03-29 and NY-03-70.

Certain nucleotide substitution patterns were observed in the RRV alignment, as summarized in Table 2. Isolates from Ontario and New York share four nucleotide substitutions: 317 G was found in all Ontario samples and two New York isolates only; 395 T is found in all Ontario and some New York samples; an A insertion at position 456 is present in two New York isolates and all Ontario isolates with two exceptions, RRV ON-99-2 and RRV ON-99-4, while no other RRV isolates share this insertion; 512 T is present in all Ontario and some New York isolates while other isolates have 512 G. All RRV isolates from New Brunswick and one isolate from New Hampshire are the only isolates to have 64 G. Nucleotide sequences of the two isolates from Quebec were identical. Five nucleotide substitutions present in the Quebec samples support direct incursion from the U.S.A.: 144 C is present only in isolates from Florida, Alabama, Georgia and Quebec; 309 A is found in isolates from Maryland, New Jersey, New Hampshire, New York and Quebec only; 320 C is present in Florida, New Jersey, New Hampshire, New York and Quebec isolates while all others have 320 T; 388 T is confined to the Quebec isolates; 503 T is shared by isolates from New Jersey, New Hampshire, Pennsylvania, New York and Quebec only.

Phylogenetic analysis

Based on phylogenetic analysis of 80 representative isolates, as identified from the alignment described above, three distinct geographically restricted lineages were identified as illustrated in the phylogenetic tree shown in Fig. 2. Lineage 1 (supported by a bootstrap value of 99%) was recovered from Florida, Alabama and Georgia. Certain viral sub-groups within this clade were supported with high bootstrap support including two clades of four FL isolates (sub-groups A and B), two clades of two FL isolates (C and D) and a group of two AL specimens (E). Lineage 2, represented by just two isolates, was found in Florida only (bootstrap value of 86%). The remaining specimens constituted lineage 3 (bootstrap value of 98%), which was broadly distributed throughout the rest of the United States (U.S.) and Canada, and exhibited only limited phylogenetic structure.

Analysis of the complete dataset of 192 sequences by the BEAST package generated a mean estimate of 7.768×10^{-4} ($\pm8.11\times10^{-6}$ SD) nucleotide substitutions per site per year. Application of this substitution rate to the RRV MCC tree (see Fig. 3), yields an estimated mean root height of 59.79 years (with 95% highest posterior density (HPD) interval of 41.44–82.07 years). This would place the date of emergence of the RRV strain at 60 years prior to the collection of the last sample, i.e. around 1946 (95% HPD range 1924 to 1965).

The MCC tree generates a phylogeny consistent with that predicted by the NJ method (Fig. 2), by providing strong support (see posterior values shown in figure) for the three lineages (1, 2 and 3) and again supporting several sub-clades within lineage 1. In particular it supports the distinctness of sub-groups A and B in Florida and additionally supports the existence of a separate southern clade composed of some Florida samples together with all specimens from Alabama and Georgia. Furthermore this tree strongly supports a dichotomy between two viral groups within lineage 3, with clade 3A represented by samples from New Jersey, New York and Quebec with two Maryland samples forming outliers to this sub-group and clade 3B represented by specimens from Virginia, West Virginia, New Hampshire, New Brunswick, Pennsylvania, New York and Ontario. The samples from New York and Ontario cluster as a separate sub-clade within clade 3B with all the Ontario samples forming a distinct branch among the New York group. In contrast the New Brunswick samples cluster most closely with a single isolate from New Hampshire.

Lineage 1 appears to be the most ancestral clade within this tree, consistent with the emergence of the raccoon strain in Florida. TMRCA for lineage 2 is predicted to date from 1968 (95% HPD 1955, 1978) and for lineage 3 around 1976 (95% HPD 1971, 1980). The viral lineage responsible for the Ontario outbreak was predicted to have emerged around 1995 (95% HPD 1992, 1995) while the distinct lineage that was responsible for the New Brunswick outbreak probably emerged around 1994 (95% HPD 1990, 1997), though all but one of the viruses of this clade were members of a lineage that emerged in 1997 (95% HPD 1992, 1999).

Spatial distribution of NY samples

The MCC tree suggested that two distinct groups of viruses were circulating within the state of New York and to explore this further the spatial distribution of all the New York cases examined here were mapped to their township of origin. The resulting map (Fig. 4) clearly illustrates that the two NY subgroups, designated NY A and NY B, exhibit geographic separation; with the exception of one isolate, all viruses of subgroup NY A occupied the south-eastern portion of the state while those of subgroup NY B were present in the western and northern regions. Also noteworthy were two New York isolates, NY-95-313 and NY-03-29, that clustered within the Ontario subgroup (Figs. 2 and 3) and which were recovered from locations relatively close to the Canada/U.S. border.

Discussion

From a public health perspective, raccoon rabies is currently one of the more important wildlife diseases in North America. The rapid spread of this disease across a large geographical area over the last 50 years has very likely been facilitated by the reservoir host's potential to reach high population densities. The importance of host population density to rabies spread was originally identified in Europe where low host population density was an important factor limiting red fox rabies spread (Steck and Wandeler, 1980) but appears to be generally applicable. Moreover since raccoons tend to inhabit urban areas they are frequently in very close proximity to humans and their domesticated animals (Rosatte, 2000). Indeed, use of human rabies post-exposure prophylaxis (PEP) increased by more than an order of magnitude following the incursion of raccoon rabies into New York in 1990 (Lee et al., 1997). Attempts to curb the spread of this disease exclusively by oral rabies vaccination (ORV) strategies in the United States have often had limited success as demonstrated by the apparent persistence of raccoon rabies in a vaccination zone in Ohio (Henderson et al., 2008) as well as its persistence in New York state. Better appreciation of the epidemiological factors contributing to epizootic spread may assist in the design of more effective control strategies. This study was therefore performed to provide improved molecular epidemiological data on the spread of this epizootic, particularly with respect to its incursion into Canada.

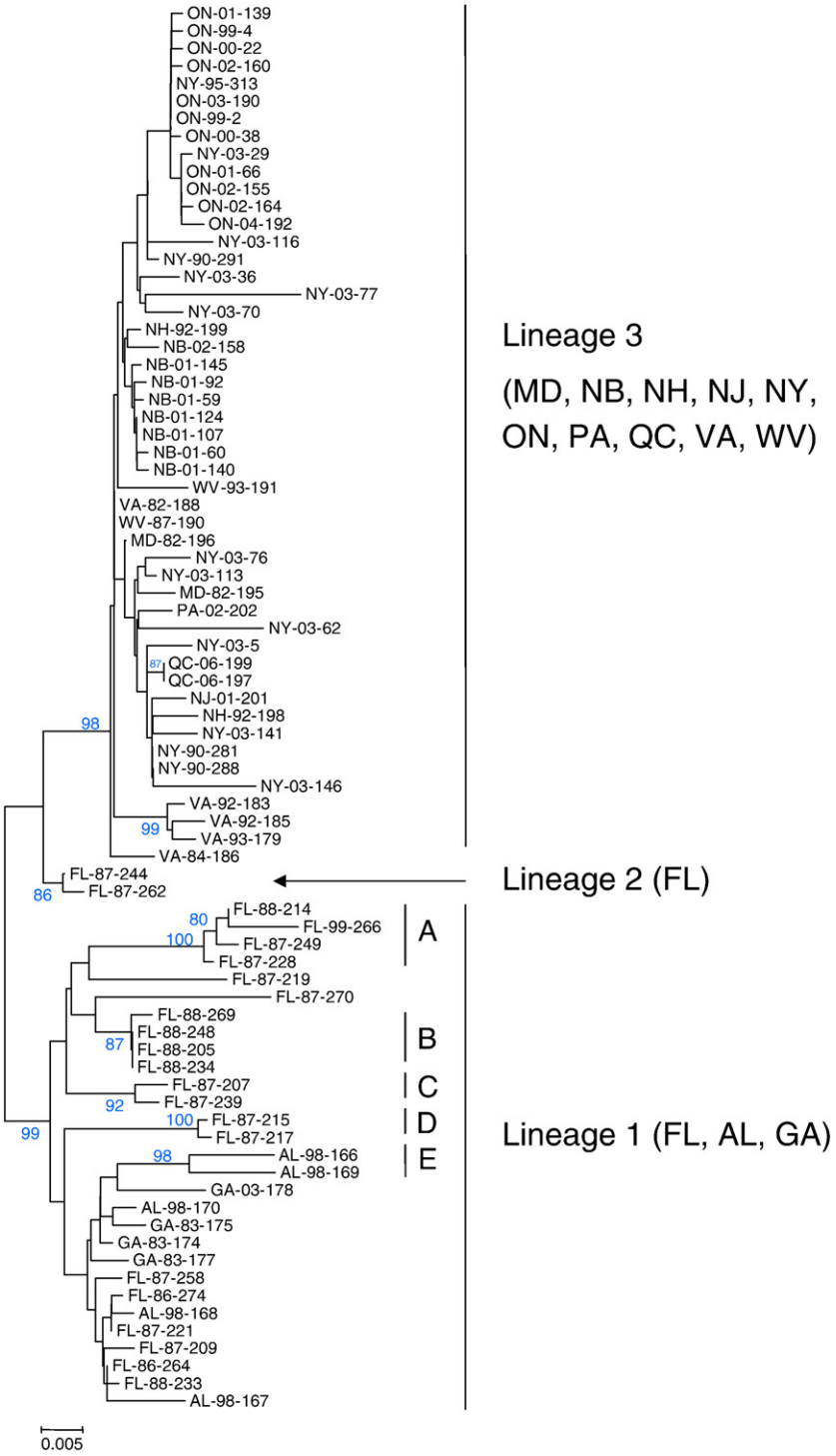
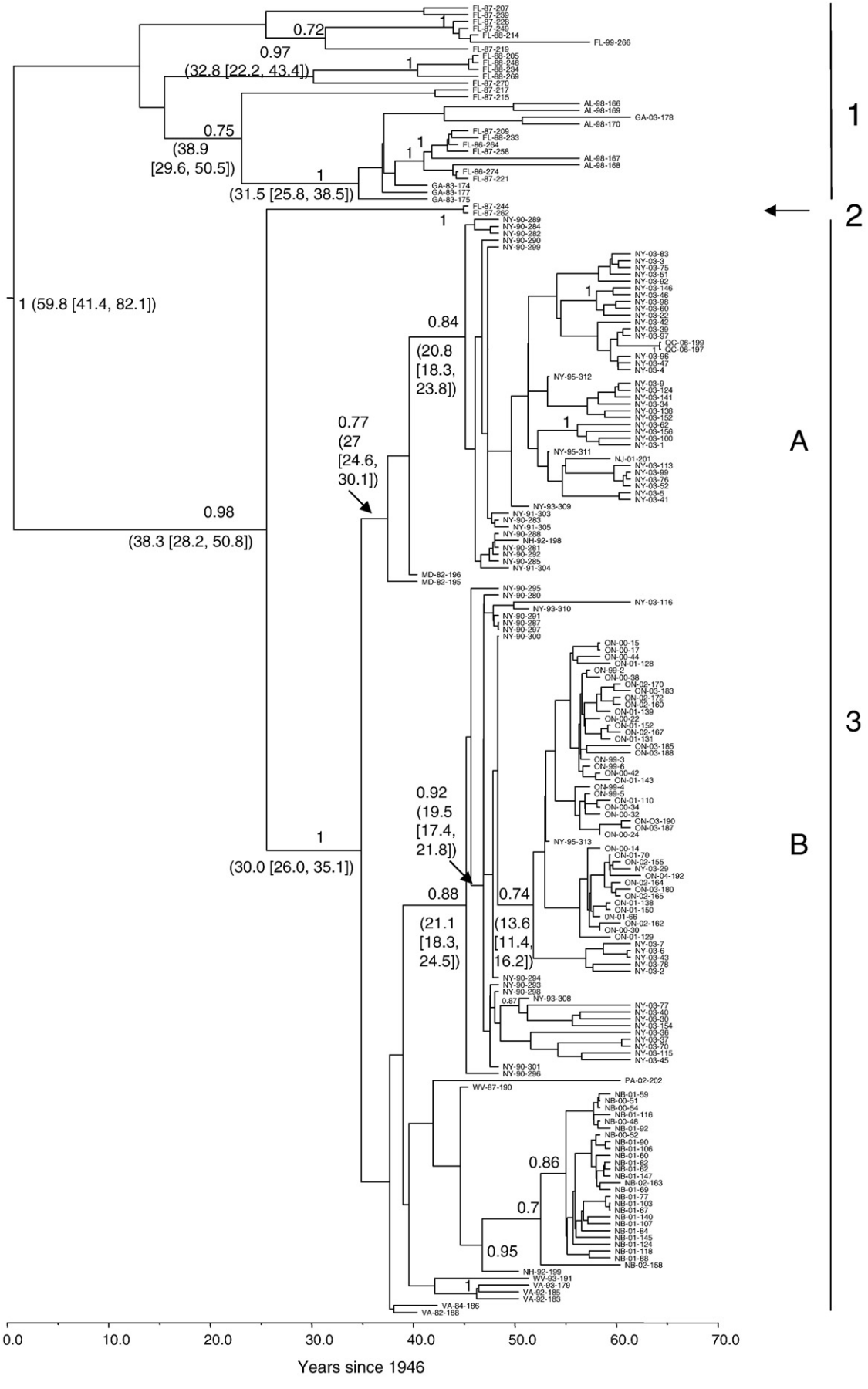


Fig. 2. Phylogenetic relationship between 80 raccoon rabies isolates in North America. Viral isolates are from nine U.S. states: Florida (FL), Alabama (AL), Georgia (GA), Virginia (VA), West Virginia (WV), Maryland (MD), New Jersey (NJ), New Hampshire (NH) and New York (NY) and the Canadian provinces of Ontario (ON), New Brunswick (NB) and Quebec (QC). The three main lineages are indicated to the right side of the tree and bootstrap values >70% are shown in blue to the left of branches.

In this study we employed the G–L non-coding region which is presumed to accumulate mutations, introduced during RNA replication through lack of proof reading/repair mechanisms (Steinhauer et al., 1992), more rapidly than coding regions. Despite this assumption the RRV G–L region was found to be highly conserved and exhibited pairwise sequence identities of ≥95%, a finding in accord with the very limited variation previously observed for the RRV P gene (Nadin-Davis et al., 2006), an RV protein coding region that is normally quite

Fig. 3. MCC tree from Bayesian coalescent analysis. Posterior values for all nodes >0.7 are shown above and to the left of the respective branches; age in years of each branch point are shown in rounded brackets below each branch together with their 95% HPD values in square brackets. The scale at the bottom represents the predicted number of years since TMRCA of the RRV strain (0 = 1946).


1
2
3
A
B
0.72
0.97
(32.8 [22.2, 43.4])
0.75
(38.9
[29.6, 50.5])
(31.5 [25.8, 38.5])
1 (59.8 [41.4, 82.1])
0.84
(20.8
[18.3,
23.8])
0.77
(27
[24.6,
30.1])
0.98
(38.3 [28.2, 50.8])
0.92
(19.5
[17.4,
21.8])
(30.0 [26.0, 35.1])
0.88
(21.1
[18.3,
24.5])
0.74
(13.6
[11.4,
16.2])
0.86
0.7
0.95
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
Years since 1946

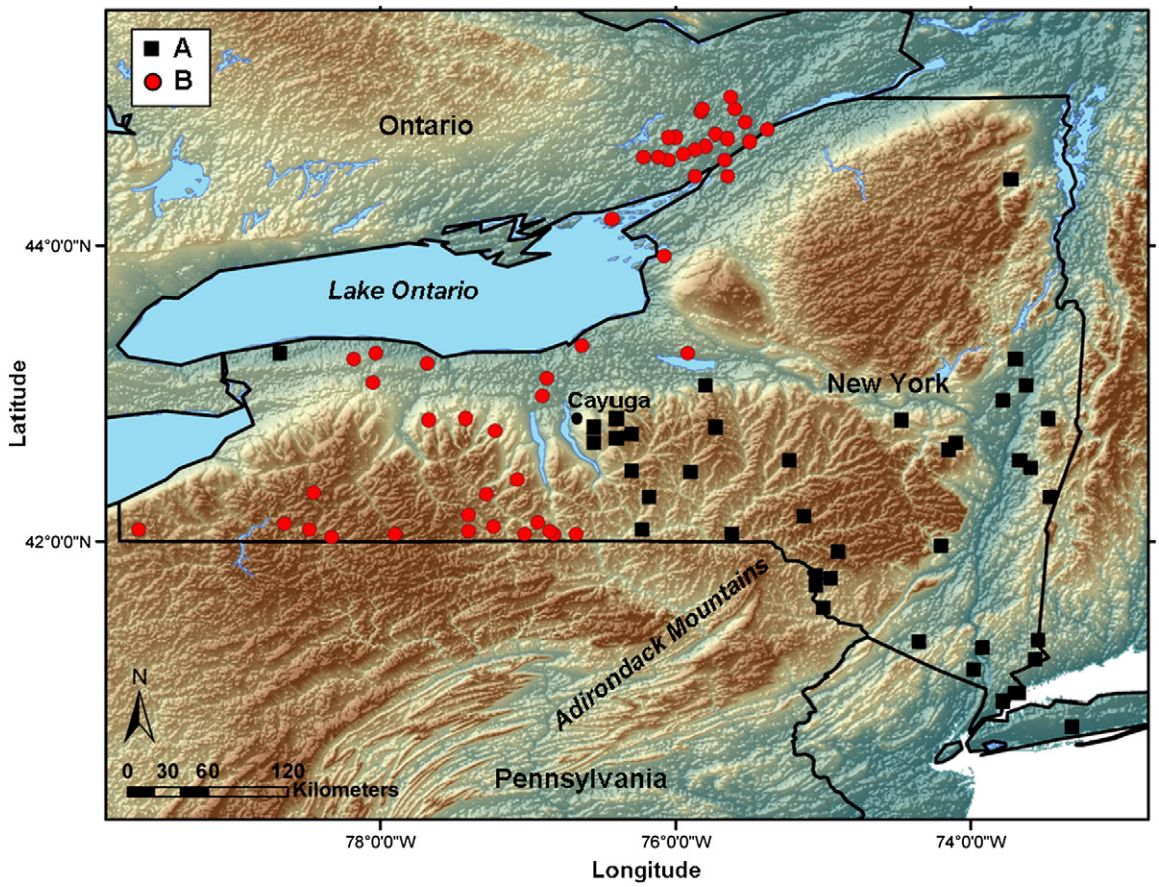


Fig. 4. Digital elevation map showing location and phylogenetic grouping of 42 Ontario and 89 New York RRV isolates. Sub-groups A and B represent the two distinct lineage 3 groupings as illustrated in Fig. 3.

variable (Nadin-Davis et al., 2002). This low genetic variability suggests either a recent emergence of this strain, consistent with surveillance data (see below) or that the genome is under strong selective pressure. Surprisingly, nucleotide substitutions in the G–L non-coding region were not randomly distributed, but four more conserved stretches of nucleotides were evident (Fig. 1). Of those, only one, the least conserved block 4, includes nucleotides with assigned functions: bases 456–465 direct G gene transcription termination and polyadenylation (TTP signal) and bases 490–498 direct transcriptional initiation of the L mRNA. Alignments of sequence data, recovered from GenBank, for the G–L region of other rabies viruses originating from different continents and from a variety of host species exhibited the same trend (data not shown), thereby demonstrating that this feature is not limited to the raccoon variant of rabies virus. Moreover, variable conservation across the G–L region was also noted during a previous study of vaccine and European fox isolates (Sacramento et al., 1992). These observations suggest that portions of the G–L non-coding region may have some regulatory function yet to be identified.

Bayesian analysis of these data estimated a rate of nucleotide substitution of 7.768×10^{-4} per site per year. This value is slightly higher than the value (2.9×10^{-4}) reported for N and G coding sequences of the RRV (Biek et al., 2007) but very close to the value of 8.26×10^{-4} reported for the G–L region of the mongoose RV variant that circulates in southern Africa (Davis et al., 2007). These values suggest that indeed the G–L region is under more relaxed constraints than the viral coding regions. The resulting MCC tree predicted TMRCA of the RRV strain to have emerged around 1946 (95% HPD values 1924, 1965), consistent with early case records documenting the first cases of raccoon rabies in Florida in 1947 (Winkler and Jenkins, 1991). This evidence supports the assumption that the RRV strain was introduced into the raccoon host during the 1940s, likely by spillover of virus from another host. Prior phylogenetic studies have indeed shown that the raccoon strain is most closely associated with the south central skunk rabies variant and that both of these strains cluster within the clade of rabies viruses that circulate in American insectivorous bats (Badrane and Tordo, 2001; Nadin-Davis et al., 2002). It is thus most likely that a spillover event from either a skunk or a bat led to the emergence of the RRV variant. The skunk is the species most commonly infected with the RRV strain after the raccoon and indeed in some areas the skunk has outnumbered the raccoon in total case reports (Hanlon et al., 2007). Such observations may suggest some role for skunks in maintenance of the RRV strain and perhaps support its emergence from the south central skunk variant. However, this theory is complicated by the lack of a major skunk epizootic in the southeast during the 1940s, and spillover from other reservoirs (e.g. bats) cannot therefore be discounted as the source of the virus that emerged into the RRV strain.

The phylogenetic analyses performed on the 192 RRV isolates of this study defined three distinct geographically restricted lineages, which are consistent with the documented history of raccoon rabies spread. Lineage 1, the ancestral raccoon strain lineage, is localized to the southeastern U.S. states of Florida, Alabama and Georgia. Moreover, this lineage could be subdivided into several sub-clades exhibiting restricted geographical ranges, particularly in Florida where the virus has circulated for the most extensive time period. The second distinct lineage which diverged from the main RRV group around 1968 (95% HPD values 1955, 1978) is represented by just two isolates from Florida; in the absence of exact location data we can only speculate that lineage 2 isolates may circulate in a region of Florida

geographically isolated from the area in which lineage 1 occurs. Lineage 3, which clearly represents the mid-Atlantic strain first documented in West Virginia in 1977 (Winkler and Jenkins, 1991), appears to have emerged from lineage 2 around 1976 (95% HPD values 1971, 1980) in good agreement with surveillance records. While NJ analysis of the sequence data obtained in this study did not strongly support extensive phylogenetic structure within lineage 3, the MCC tree clearly supported some spatial clustering as the epidemic moved northwards; in particular this study identified two genetically distinct and geographically separated subgroups within lineage 3. This dichotomy was most notable within the New York sample set where spatial restriction of two viral subgroups, NY A and NY B was identified (Fig. 4). All subgroup NY A viruses were located exclusively in the southeastern corner of the state with the exception of one isolate, found in the far western region of the state and which we speculate was the result of translocation of a diseased animal from the southeastern area. Subgroup NY B viruses occupied the western and northern regions with the Finger Lakes area apparently forming a barrier to the western movement of the viruses of subgroup NY A; the town of Cayuga located within the Finger Lakes region was the only location from which both viral variants were recovered. In the north of the state, the Adirondacks appear to have formed a major barrier to the northward expansion of subgroup NY A viruses, and indeed relatively few cases of raccoon rabies have been reported north of this range. Those that have occurred represent subgroup NY B viruses; this variant most likely reached the northernmost parts of the state via northeastward movement through country near to the southern shore of Lake Ontario prior to crossing the St. Lawrence River to enter Ontario. This variant distribution is consistent with the pattern of invasion of New York State by RRV in the early 1990s. The first cases were reported from the southeastern corner of the state due to incursion from either eastern Pennsylvania or neighboring eastern states, whereas later a wave of invasion entered western New York from western Pennsylvania (Biek et al., 2007).

The clustering patterns of the samples from the three Canadian outbreaks clearly suggest their independent origins, arising most likely through cross-border incursion from the U.S. in each case. The close relationship between New Brunswick and New Hampshire isolates suggests that the New Brunswick incursion came from the northeastern U.S., likely from the neighboring state of Maine (MacInnes, 2000; Nadin-Davis et al., 2006). The viral progenitor of the New Brunswick outbreak is estimated to have emerged as early as 1994 (95% HPD values 1990, 1997), some years prior to the first recorded case in 2000. It remains possible that a small focus of infection remained undetected in the province over this time period because of limited surveillance. However, all but one New Brunswick sample formed a discrete cluster with a later date of emergence (estimated at 1997 with 95% HPD values 1992, 1999), and it is possible that this lineage was responsible for most of the outbreak while a separate lineage, corresponding to the single outlier (NB-02-158), had emerged on the U.S. side of the border and was responsible for a second later incursion into the province.

The Ontario isolates form a single homogeneous cluster that emerged from the NY B subgroup of viruses, thus confirming early reports of rabies movements across the New York/Ontario border (Rosatte et al., 2001, 2006, 2007a). The year of emergence of TMRCA of the Ontario group is estimated at 1995 (95% HPD values 1995, 1997), four years prior to the first recorded case in 1999, and this variant may have circulated in northern New York State for some time prior to cross-border incursion. Indeed isolate NY 95-313, recovered from Jefferson County near the St. Lawrence River, is probably closely related to the virus that was responsible for the Ontario incursion given its close genetic similarity to several of the early isolates of the Ontario outbreak and its isolation just a few years prior to the Ontario outbreak. It carries the A to G substitution at position 317 typical of all Ontario isolates (see Table 2). The other New York virus that grouped within the Ontario cluster, NY03-29, is most closely related to Ontario viruses circulating in Leeds County in 2001 and 2002, and bears both the A to G substitution at position 317 as well as the A insertion at position 465. We suggest that this variant may have crossed back into New York State from Ontario where it was detected in a skunk in Oswego County. In fact, Rosatte et al. (2007a) documented the movement of raccoons from Ontario to New York State during 1997 to 2005. The other New York sample to carry the A insertion at position 465 was sample NY-03-70, which clustered within the NY B subgroup well outside the Ontario clade; this insertion event would appear to be independent of the one that occurred very early during the Ontario incursion and became established throughout the viruses of the Ontario outbreak. This latter observation clearly emphasizes the difficulty in drawing conclusions as to variant origins based on single nucleotide changes in the absence of additional phylogenetic evidence.

While only two Quebec samples were included in this study, these isolates were typical of the outbreak in this province (data to be presented elsewhere) and their clustering with the distinct NY A subgroup is consistent with this outbreak having originated from either the eastern part of New York or the neighboring state of Vermont, and not from a Canadian source. The emergence of the Quebec lineage in 2006 is consistent with the first two case reports in 2006.

A previous analysis of a collection of RRVs from the US focusing on the coding regions of the N and G genes (Biek et al., 2007) identified a total of 7 lineages: southeastern (SE), southwestern (SW), eastern (E), northeastern NE1 and NE2, northwestern NW1 and NW2 (Biek et al., 2007). Based on the regional distribution of the clades identified in our study as the NY A and NY B subgroups and a separate northeastern clade composed mainly of New Brunswick samples, it would appear likely that they represent the NE2, NW1 and NE1 lineages respectively. The additional lineages proposed by Biek et al. (2007) are likely represented in this study by small numbers of isolates from areas only lightly sampled; e.g., two Maryland samples may be equivalent to the E lineage while the West Virginia and Virginia samples likely represent the SE, SW and NW2 lineages. In this study the inclusion of samples from southern states identified an additional RRV lineage.

The high costs of rabies control and prevention (Recuenco et al., 2007) can be minimized, by employing the most appropriate strategies that take into account rabid raccoon movements that lead to viral spread (Rosatte et al., 2006, 2008, 2009a). Two distinct mechanisms of RRV spread have been identified. Most commonly localized contiguous epizootic spread is due to animal-to-animal contact. However, long-distance translocation of infected animals, which can introduce disease into areas some distance away from a disease front, can also occur (see Smith et al., 2005), either as the result of deliberate human-assisted acts (which first introduced the raccoon strain into West Virginia), or inadvertently when animals hitch rides on long-distance transporters (Rosatte et al., 2007a, b). Indeed, exceptional movements (mean of 479 km) of raccoons riding in vehicles from the United States into Ontario have been documented (Rosatte et al., 2007a). Different control strategies are required to combat these two distinct scenarios. Local epizootic spread, including breach of natural geographic barriers, is best controlled by generating immune population barriers by activities such as trap-vaccinate-release (TVR) or ORV (Rosatte et al., 1992, 2008, 2009a, b). Where disease foci occur in previously naïve areas, the most effective control strategy is point infection control (PIC) (Rosatte et al., 2001) coupled with increased inspection of vehicular traffic, especially transport trucks, which can accidentally transport rabid animals beyond the controlled zone (Rosatte et al., 2007a). Since this study shows that in Canada all three epizootics were genetically distinct and exhibited characteristics most consistent with their introduction from a local source, i.e. neighboring U.S. states, application of TVR, PIC and ORV in graded responses was the most effective strategy in all three

provinces. As a result of an effective rabies control and management program in Canada undertaken by provincial authorities, with particular leadership by the Ministry of Natural Resources in Ontario, there has been no viral spread between the three provinces.

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