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## MOLECULAR EPIDEMIOLOGY OF TERRESTRIAL RABIES IN THE FORMER SOVIET UNION

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**ABSTRACT:** Fifty-five rabies virus isolates originating from different regions of the former Soviet Union (FSU) were compared with isolates originating from Eurasia, Africa, and North America according to complete or partial nucleoprotein (N) gene sequences. The FSU isolates formed five distinct groups. Group A represented viruses originating from the Arctic, which were similar to viruses from Alaska and Canada. Group B consisted of “Arctic-like” viruses, originating from the south of East Siberia and the Far East. Group C consisted of viruses circulating in the steppe and forest-steppe territories from the European part of Russia to Tuva and in Kazakhstan. These three phylogenetic groups were clearly different from the European cluster. Viruses of group D circulate near the western border of Russia. Their phylogenetic position is intermediate between group C and the European cluster. Group E consisted of viruses originating from the northwestern part of Russia and comprised a “northeastern Europe” group described earlier from the Baltic region. According to surveillance data, a specific host can be defined clearly only for group A (arctic fox; *Alopex lagopus*) and for the Far Eastern part of the group B distribution area (raccoon dog; *Nyctereutes procyonoides*). For other territories and rabies virus variants, the red fox (*Vulpes vulpes*) is the main virus reservoir. However, the steppe fox (*Vulpes corsac*), wolf (*Canis lupus*), and raccoon dog are also involved in virus circulation, depending on host population density. These molecular data, joined with surveillance information, demonstrate that the current fox rabies epizootic in the territory of the FSU developed independently of central and western Europe. No evidence of positive selection was found in the N genes of the isolates. In the glycoprotein gene, evidence of positive selection was strongly suggested in codons 156, 160, and 183. At these sites, no link between amino acid substitutions and phylogenetic placement or specific host species was detected.

**Key words:** Epidemiology, former Soviet Union, rabies, phylogenetics, Russia.

### INTRODUCTION

Rabies is enzootic in Eurasia. The Asian tropics are affected mainly by urban dog rabies, and thousands of human cases are reported there annually (Ahuja et al., 1985; Wilde, 1997). In Europe, urban dog rabies has been largely eliminated. The main wildlife reservoir is the red fox (*Vulpes vulpes*), with only sporadic human cases registered in European countries over the last few decades, mainly because of successfully implemented oral vaccination programs and advanced human postexposure prophylaxis (Toma and Andral, 1977; Bourhy et al., 1999). In the former Soviet Union (FSU), rabies is enzootic from the western borders to the Far East, but the affected areas are discontinuous (Fig. 1).

General epidemiologic patterns of the disease, surveillance data, and veterinary records suggest that rabies is maintained in Russia by wild canids. During the last several decades, only a few local dog epizootics have occurred sporadically in some territories. Wildlife rabies predominates also in the Ukraine, Belarus, Moldova, and the Baltic states. Dog rabies is predominant in the central Asiatic states of the FSU, especially in Uzbekistan and Tajikistan and in the Caucasus (Cherkasskiy, 1985; Selimov, 1998; Vedernikov et al., 2002).

Certain wild Carnivora species appear to maintain rabies virus circulation in different regions. Arctic rabies covers the tundra and tundra-forest zones in the

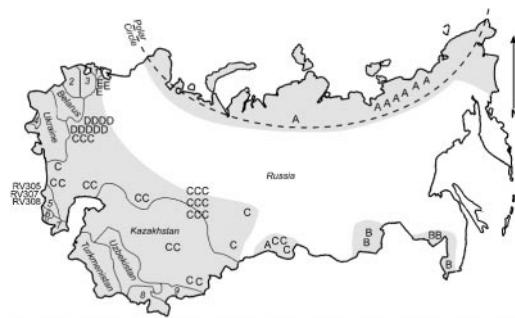


FIGURE 1. Map of the former Soviet Union showing the terrestrial rabies enzootic region (shaded) and distribution of different phylogenetic groups of rabies virus (A-E). State designation: 1 = Estonia; 2 = Lithuania; 3 = Latvia; 4 = Moldova; 5 = Georgia; 6 = Armenia; 7 = Azerbaijan; 8 = Tajikistan; 9 = Kyrgyzstan. RV305, RV307, and RV308 = ungrouped isolates from Georgia.

north, and this enzootic region matches the area of distribution and regular migrations of the arctic fox (*Alopex lagopus*). A wide zone of conifer taiga forests lying to the south of the tundra-forest zone is considered largely free of rabies. In general, this region is not supportive of wild canids, and their density appears too low to maintain active virus circulation. In the forest-steppe and steppe zones, lying to the south of the taiga, rabies is maintained primarily by the red fox. The raccoon dog (*Nyctereutes procyonoides*) is involved in virus circulation in the Far East and parts of Europe. The steppe fox (*Vulpes corsac*) and jackal (*Canis aureus*) participate in rabies virus circulation in the steppe and desert territories. The wolf (*Canis lupus*) historically caused rabies outbreaks in different areas. These six canid species were included in the group of likely principal hosts and vectors of rabies virus in Russia and the FSU. Mustelids and other carnivores were considered as additional or occasional hosts (Kantorovich and Reshetnikov, 1968; Selimov, 1978; Malkov and Gribova, 1980). Rodents and other terrestrial mammals have been diagnosed rabid, but only very rarely (Selimov, 1998; Vedernikov et al., 2002).

At least nine different antigenic variants

of rabies virus were detected in the FSU using antinucleocapsid monoclonal antibodies (N-MAbs). A limited relationship of the antigenic patterns to geographic origin and terrestrial host species of the isolate was demonstrated (Botvinkin et al., 1990; Selimov et al., 1994).

Few isolates originating from Russia and states of the FSU were available for genetic comparison. Comparative analyses of some of these isolates from the FSU have been published, but not with specific attention to the FSU (Kissi et al., 1995; McElhinney et al., 2001; Nadin-Davis et al., 2002; Johnson et al., 2003). In this study, we performed phylogenetic analysis of 55 terrestrial rabies virus isolates, originating from different geographic locations of Russia and some surrounding states of the FSU, and compared them with isolates originating from Eurasia, Africa, and North America. Our overall objective was to draw inferences into the origins, reservoirs, and regional epidemiology of the disease, as well as to search for comparative evidence of selection among viral genes.

## MATERIALS AND METHODS

All viruses were isolated from rabid animals by intracerebral mouse inoculation (Koprowski, 1996) and were used after a minimum of one to seven mouse brain passages (Table 1). The samples were initially identified by the direct fluorescent antibody test (Dean et al., 1996) or the rapid rabies enzyme immunoassay (Bourhy and Perrin, 1996).

Total RNA was extracted from infected mouse brains with TRIzol® (Gibco-BRL, Inc., Gaithersburg, Maryland, USA) according to the manufacturer's recommendations. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with primer sets to the nucleoprotein (N) and glycoprotein (G) genes and sequenced as described elsewhere (Tordo et al., 1996; Johnson et al., 2003).

Two data sets were used for the phylogenetic analysis. The first set ( $n=74$ , including 39 isolates from the FSU) consisted of 1,350 nucleotide (nt) sequences of the entire N gene (positions 71–1,420 according to the Pasteur virus [PV] genome; GenBank accession number M13215). The second set ( $n=92$ , including 55 isolates from the FSU) consisted of 405 nt se-

TABLE 1. Description of viruses used for study of the molecular epidemiology of rabies in the former Soviet Union.

Country	Region/Province	Species	Isolate	Phylogroup <sup>a</sup>	Source/reference	GenBank accession no. <sup>b</sup>	
						N	G
Russia	Altay	Red fox ( <i>Vulpes vulpes</i> )	3502f	C	This study	AY352455	AY353857
	Belgorod	Cat	RV1589	C	This study	AY352456	AY353858
	Bryansk	Red fox	RF262	D	This study	AY352457	AY353859
	Chabarovsk	Raccoon dog ( <i>Nyctereutes procyonoides</i> )	857r	B	This study	AY352458	AY353860
	Chabarovsk	Human	RV304	B	This study	AY352502	
	Chita	Steppe fox ( <i>Vulpes corsac</i> )	304c	B	This study	AY352459	AY353861
	Chita	Steppe fox	248c	B	This study	AY352460	AY353862
	Krasnodar	Red fox	1305f	C	This study	AY352461	AY353863
	Krasnodar	Cat	RV290	C	This study	AY352503	
	Krasnoyarsk	Human	RVHK	A	This study	AY352462	AY353864
	Leningrad	Raccoon dog	RV309	E	This study	AY352504	
	Novosibirsk	Human	RVHN	C	This study	AY352463	
	Omisk	Red fox	RV257	C	This study	AY352464	AY353865
	Omisk	Red fox	RV260	C	This study	AY352465	AY353866
	Omisk	Red fox	3665f	C	This study	AY352466	AY353867
	Omisk	Red fox	3605f	C	This study	AY352467	AY353868
	Omisk	Steppe fox	3678c	C	This study	AY352468	AY353869
	Omisk	Steppe fox	3683c	C	This study	AY352469	AY353870
	Omisk	Red fox	3687f	C	This study	AY352470	AY353871
	Omisk	Cow	3454cow	C	This study	AY352471	AY353872
	Orenburg	Human	RV1590	C	This study	AY352472	AY353873
	Orenburg	Cat	999c	C	This study	AY352473	AY353874
	Primorie	Raccoon dog	RV303	B	This study	AY352505	AY353875
	Pskov	Red fox	RV1596	E	This study	AY352474	AY353876
	Pskov	Human	RV245	E	This study	AY352475	AY353877
	Tula	Dog	RV234	D	This study	AY352476	
	Tula	Human	RV241	C	This study	AY352477	AY353878
	Tula	Cow	RV298	C	This study	AY352478	AY353879
	Tula	Red fox	RV299	D	This study	AY352479	AY353880
	Tula	Red fox	RV236	D	This study	AY352506	

TABLE 1. Continued.

Country	Region/Province	Species	Isolate	Phylogroup <sup>a</sup>	Source/reference	GenBank accession no. <sup>b</sup>	
						N	G
Kazakhstan	Tula	Dog	RV237	D	This study	AY352507	
	Tula	Dog	RV238	D	This study	AY352508	
	Tula	Human	RV239	D	This study	AY352509	
	Tula	Red fox	RV246	C	This study	AY352510	
	Tula	Red fox	RV247	D	This study	AY352511	
	Tuva	Rodent ( <i>Citellus undulatus</i> )	RV250	A	This study	AY352480	AY353881
	Tuva	Dog	3561d	C	This study	AY352481	AY353882
	Tuva	Cow	686cow	C	This study	AY352482	AY353883
	Tuva	Wolf ( <i>Canis lupus</i> )	765w	C	This study	AY352483	AY353884
	Volgograd	Red fox	2070f	C	This study	AY352484	AY353885
Saudi Arabia	Volgograd	Red fox	2072f	C	This study	AY352485	AY353886
	Yakutia	Wolf	3510w	A	This study	AY352486	AY353887
	Yakutia	Arctic fox	483a	A	This study	AY352487	AY353888
	Yakutia	Arctic fox	743a	A	This study	AY352488	AY353889
	Yakutia	Arctic fox	RV251	A	This study	AY352512	
	Yakutia	Reindeer ( <i>Rangifer tarandus</i> )	RV293	A	This study	AY352513	
	Yakutia	Arctic fox	RV294	A	This study	AY352514	
	Almaty	Red fox	409f	C	This study	AY352489	AY353890
	Almaty	Sheep	408s	C	This study	AY352490	AY353891
	Celinograd	Red fox	RV259	C	This study	AY352491	AY353892
Sultanate of Oman	Celinograd	Red fox	341f	C	This study	AY352492	AY353893
		Red fox	8706ARS		Kissi et al., 1995	U22481	
		Red fox	9135OMIA		Kissi et al., 1995	U22480	
		Human	8738THA		Kissi et al., 1995	U22653	
	India	Human	RV61		This study	AY352493	AY353894
		Dog	8681IRA		Jayakumar (unpubl.)	AY374721	
	Iran	Dog	8702IRA		Kissi et al., 1995	U22482	
		Wolf			Kissi et al., 1995	U22483	
	Tokyo	Dog	Komatsugawa		This study	AY352494	AY353895
	Pakistan	Cow	196p		This study	AY352495	AY353896
Georgia		Goat	277p		This study	AY352496	
		Dog	RV305	UG	This study	AY352515	

TABLE 1. Continued.

Country	Region/Province	Species	Isolate	Phylogroup <sup>a</sup>	Source/reference	GenBank accession no. <sup>b</sup>	
						N	G
Turkey		Cow	RV307	UG	This study	AY352516	
		Human	RV308	UG	This study	AY352497	AY353897
		Dog	RV1133		Johnson et al., 2003	AY091619	
Estonia		Raccoon dog	9339EST	E	Bourhy et al., 1999	U42707	AF134335
		Raccoon dog	9342EST	E	Bourhy et al., 1999	U43432	AF134336
		Raccoon dog	9142EST	E	Bourhy et al., 1999	U22476	AF134339
France		Red fox	9244FRA		Bourhy et al., 1999	U42607	AF134342
		Red fox	9223FRA		Bourhy et al., 1999	U43433	AF134326
		Red fox	9212ALL		Kissi et al., 1995	U22475	AF134337
Germany		Red fox	9202ALL		Bourhy et al., 1999	U42701	AF134338
		Human	9215HON		Bourhy et al., 1999	U43025	AF134343
		Wolf	8653YOU		Bourhy et al., 1999	U42704	AF134341
Hungary		Red fox	86111YOU		Bourhy et al., 1999	U42706	
		Red fox	86107YOU		Bourhy et al., 1999	U42703	AF134345
		Red fox	8658YOU		Bourhy et al., 1999	U42705	AF134344
Ethiopia		Cow	8807ETH		Kissi et al., 1995	U22637	
		Hyena ( <i>Crocuta crocuta</i> )					
Algeria		Dog	9137ALG		Kissi et al., 1995	U22643	
		Human	8721AFS		Kissi et al., 1995	U22633	
		Dog	4795		This study	AY352498	AY353898
South Africa	Alaska	Red fox	1420		This study	AY352499	
		Red fox	1421		This study	AY352500	AY353899
		Red fox	1422		This study	AY352501	AY353900
Canada	Alaska	Arctic fox	4055DG		Nadin-Davis et al., 1994	U03770	
		Dog	8480FX		Nadin-Davis et al., 1994	U03768	
		Red fox	1090DG		Nadin-Davis et al., 1994	U03769	
Laboratory strain		Dog	1991 fox 6199		Nadin-Davis et al., 1994	U11734	
		Red fox	91RABN1035		Nadin-Davis et al., 1994		U11736
		Arctic fox	91RABN0783		Nadin-Davis et al., 1994		U11757
		Red fox	CVS		Tordo et al., 1986	D42112	
					Prehaud et al., 2003		
					Conzelmann et al., 1990	M31046	AF406694
			SAD B19				M31046

<sup>a</sup> Phylogroups are shown for viruses originating from the former Soviet Union only. UG = ungrouped.<sup>b</sup> N = nucleoprotein; G = glycoprotein.



quences of the N gene (positions 71–475). Phylogenetic analysis was performed by the neighbor-joining (NJ) method from the MEGA computer program, version 2.1 (Kumar et al., 2001). Maximum likelihood (ML) analysis, with empirical base frequencies, gamma distribution of rate variation among sites, and the hidden Markov model of inferring different rates of evolution at different sites, was performed by the method implemented in PHYLIP, version 3.6-alpha (Felsenstein, 1993). Initially, the data set was subjected to multiple replications in the SEQBOOT module. The obtained file was processed subsequently in the DNAPARS, PROTPARS, or DNAML modules, respectively, and finally, the consensus tree was generated with the CONSENSE module. Bootstrap values were determined for 1,000 replicates by the NJ method and for 100 replicates by the ML method.

Three data sets were used for positive selection analysis: 1) entire N gene sequences ( $n=67$ ); 2) 852 nt G gene sequences ( $n=36$ ; 284 codons, positions 3,408–4,259); and 3) 251 nt G gene sequences ( $n=52$ ; 117 codons, positions 4,009–4,259). Positive selection analysis was performed with various models of codon substitution (Yang et al., 2000) implemented in the CODEML program of the PAML package (Yang, 1997). Various ML models are applied with varying constraints on the values of synonymous ( $d_S$ ) and nonsynonymous ( $d_N$ ) substitution rates, and their ratio ( $\omega$ ). Models allowing for positive selection (i.e.,  $\omega>1$ ) are nested within models that do not allow for positive selection. This allows the significance of the fit for positive selection models to be tested by the likelihood ratio test. Positive selection is inferred if the positive selection model has a significantly higher likelihood than the null model, and a value of  $\omega>1$  is estimated. If evidence of positive selection is suggested, Bayesian methods are used to identify which individual codons fall into the  $\omega>1$  class.

Sequence alignments were trimmed to include only complete nonstop codons, and identical sequences were removed. For each set of sequences, a ML tree was generated with PAUP\* 4.0, beta 10 (Swofford, 2000) by a heuristic search incorporating tree bisection-recombination (TBR) branch swapping. This tree was then used for positive selection analysis. In each case, the model of nucleotide substitution was selected with Modeltest (Posada and Crandall, 1998). Model testing with CODEML was performed as described by Woelk et al. (2002). Phylogenetic trees used in this analysis are available from the authors on request.

## RESULTS

Phylogenetic analysis of the entire N gene sequences, performed by either ML or NJ methods, revealed trees of an identical topology (Fig. 2). The sequences were subdivided into two main groups. The first group consisted of two tropical dog viruses from Thailand (8738THA) and India (AF374721) and might be used as an outgroup to the second group, which consisted of all other sequences. The second group was further subdivided into two distinct clusters. One of them (cluster I) joined Arctic viruses originating from Eurasia and North America and two branches of “Arctic-like” viruses, originating from India, Pakistan, East Siberia, and the Far East (bootstrap support of 100%). The second (cluster II) contained viruses originating from different territories of Eurasia and Africa (bootstrap support of 99%). Included in this cluster were representatives of all European groups previously identified (Bourhy et al., 1999) and other viruses of Old World origin (Kissi et al., 1995; McElhinney et al., 2001). The topology of the tree for all these sequences matched that of earlier reports.

Isolates originating from the FSU could be clearly subdivided into five main groups on the basis of phylogenetic placement. Diversity varied primarily with geographic location rather than host species, even when a number of isolates from different species were available for comparison. Group A consisted of isolates originating from the Arctic zone of Eurasia and North America. This cluster was further split into two clades, and the Canadian isolate “1991 fox 6199” was placed ancestral to both. No difference was registered among these Arctic viruses with regard to continent of origin or host species, with one exception: the isolate RV250, obtained from a rodent, *Citellus undulatus*, in the south of East Siberia, distant from the Arctic zone.

Group B consisted of Arctic-like viruses originating from East Siberia and the Far East, including the Japanese isolate, Ko-

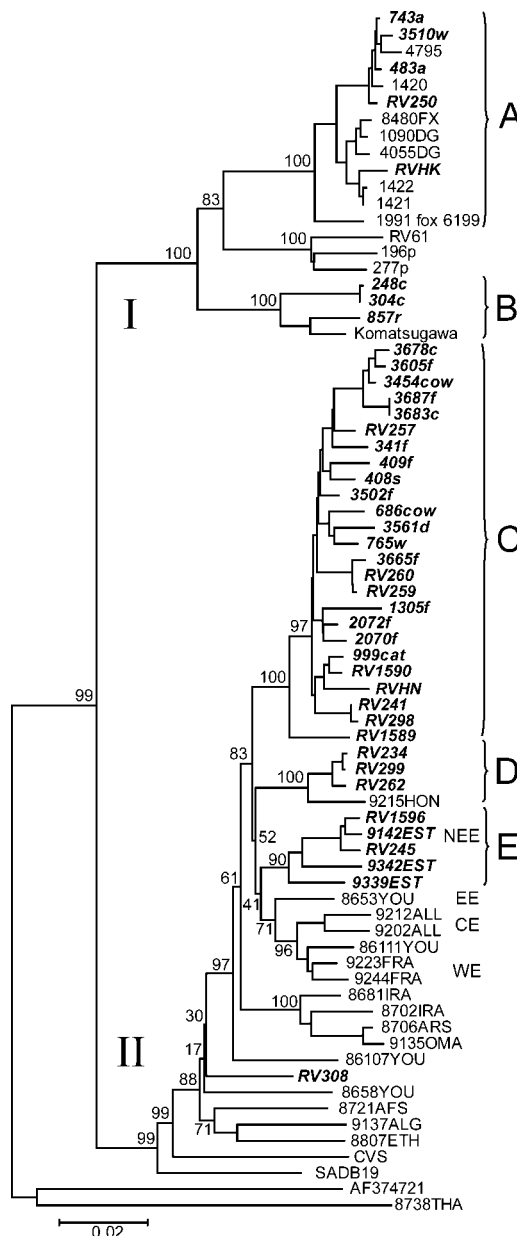


FIGURE 2. Neighbor joining phylogenetic tree of 74 rabies virus isolates according to the entire nucleoprotein gene. Genbank accession numbers are given when strain names are unavailable. The tree is rooted with the isolates AF374721 and 8738THA. Bootstrap values are presented for key nodes, and branch lengths are drawn to scale. Isolates from the former Soviet Union are italicized. Abbreviations for the main phylogenetic groups from the former Soviet Union (A–E) are described in the text, as well as big clusters (I and II); abbreviations for earlier described European groups (NEE, CE, EE, WE) are given according to Bourhy et al. (1999).

matsugawa. Another Arctic-like group included viruses originating from India and Pakistan (isolates RV61, 196p, and 277p). Group C consisted of viruses originating from the steppe and forest-steppe territories from the European part of Russia to the Altai and Sayan mountains. Differences were well supported from the other group of viruses, which joined isolates from different parts of Europe (Kissi et al., 1995; Bourhy et al., 1999), with bootstrap support of 100%.

Group D joined viruses originating from the center of the European part of Russia (RV234, RV299, and RV262) and an isolate from Hungary, 9215HON, which was not related to any clade from an earlier study (Bourhy et al., 1999). This group was linked to a cluster of European viruses with limited bootstrap support (52%). Group E consisted of viruses isolated in the northwestern part of Russia. Together with isolates from Estonia, these viruses belonged to the earlier described “northeastern Europe” group (Bourhy et al., 1999). The isolate RV308 from Georgia was not classified into any cluster.

When the extended set of 405 nt N gene sequences was analyzed, tree topology was identical. All virus groups determined for the entire N gene remained and were extended with the new representatives, whereas bootstrap support was reduced (Fig. 3). In this tree, the newly incorporated isolates RV305 from Georgia and RV1133 from Turkey were joined to isolate RV308 and formed a solid group with substantial bootstrap support. Another newly incorporated isolate from Georgia, RV307, was joined to the cluster of Middle East viruses.

No evidence of positive selection was found in any set of N gene sequences. In a number of cases, the null model was significantly less likely than one that allowed positive selection, but in no case was  $\omega > 1$ . The results of positive selection analysis for G gene sequences are shown in Table 2. For both G gene data sets, a number of null models were less favored than models



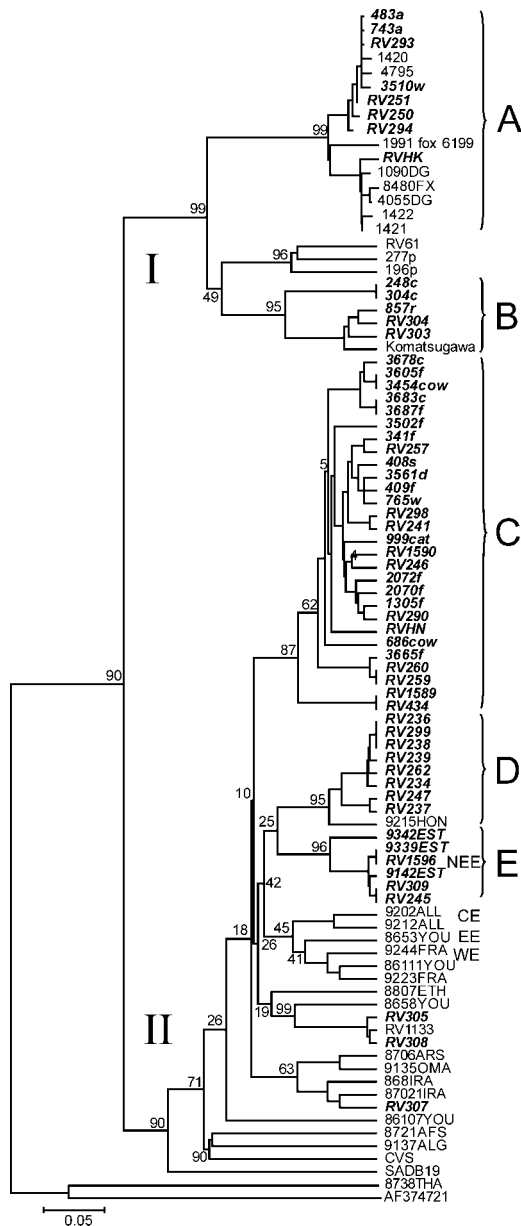


FIGURE 3. Neighbor joining phylogenetic tree of 92 rabies virus isolates according to 405 nucleotide fragment of the nucleoprotein gene. For explanations see legend to Figure 2.

that allowed for positive selection. In both cases, this includes the most precise model (M8) for detecting positive selection (Anisimova et al., 2002). For the sequence set covering the larger area of the G gene (data set 2), two positive selection models

(M3 and M8) selected three codons (156, 160, 183) as having a  $\omega=2.2709$  and 1.7427, respectively. For the smaller sequence set (data set 3), positive selection was inferred at just one codon (183) by M3 and M8, with  $\omega=2.3301$  and 2.3266, respectively.

## DISCUSSION

Geographic distribution of the genetic variants of rabies virus is presented in Figure 1. The isolates belonging to groups A–E originated from distinct geographic regions. Arctic rabies is distributed in a circumpolar fashion. Absence of significant distinctions between North American and Siberian Arctic rabies virus isolates suggests that the virus population is intermixed. This is in concordance with the results of N-MAbs application (Botvinkin et al., 1990) and previous genetic data (Kissi et al., 1995). Our set of sequences was limited, and we could not make a species comparison. However, surveillance data suggests that the arctic fox is the principal host of rabies virus in the Arctic region of Russia. Rare human rabies cases caused by arctic foxes, dogs, and wolves were described in northern Russian (Kantorovich and Reshetnikov, 1968; Botvinkin et al., 1995; Kuzmin, 1999). Additional attention should be paid to isolate RV250. The isolation point of this virus is separated from the native area of Arctic rabies virus circulation by at least 1,000 km of the “rabies-free” zone of the taiga forests. Further active surveillance of *C. undulatus* and another rodent species did not bring positive results. According to antigenic typing, this isolate reacted with N-MAb P-41, like the Arctic rabies viruses, but did not react with some other N-MAbs, being different from all other rabies viruses ( $n>300$ ) available for comparison (Botvinkin et al., 1990). Isolates with similar antigenic patterns were described from Thailand and Madagascar (Sureau et al., 1983). We have no satisfactory explanation for the origin of this virus.

Circulation of group B viruses in the

TABLE 2.. Results of positive selection analysis

Data set <sup>a</sup>	Model <sup>b</sup>	<i>P</i> values <sup>c</sup>	$\omega$ <sup>d</sup>	<i>P</i> <sup>e</sup>	Codons <sup>f</sup>
2	M2	< <b>0.0001</b> (M0), < <b>0.0001</b> (M1), 0.4161 (M3)	0.037	0.734	
	M3	< <b>0.0001</b> (M0), < <b>0.0001</b> (M1), 0.4161 (M2)	2.271	0.006	156, 160, 183
	M8	< <b>0.0001</b> (M7)	1.743	0.011	156, 160, 183
3	M2	< <b>0.0001</b> (M0), 0.3991 (M1), 0.4161 (M3)	3.583	0.006	
	M3	< <b>0.0001</b> (M0), < <b>0.0001</b> (M1), < <b>0.0001</b> (M2)	2.330	0.009	183
	M8	<b>0.0006</b> (M7)	2.327	0.009	183

<sup>a</sup> Data set 2 = long glycoprotein (G) gene (n = 36; 284 codons); data set 3 = short G gene (n = 52; 117 codons).

<sup>b</sup> Refers to the model allowing for positive selection implemented with the CODEML package. Parameters for all models are available from the authors on request.

<sup>c</sup> Significance of the likelihood ratio test performed as described by Woelk et al. (2002). Bold values indicate significant improvement on the null hypothesis by a model allowing for positive selection.

<sup>d</sup> Estimated value of the ratio of synonymous ( $d_S$ ) and nonsynonymous ( $d_N$ ) substitution rates.

<sup>e</sup> Proportion of codons estimated to fall into the category of  $\omega$ .

<sup>f</sup> Position of selected codons along the rabies virus glycoprotein gene.

Russian Far East is currently maintained by raccoon dogs, red foxes, and wolves. According to available surveillance data, rabid raccoon dogs have been identified in the Far East since 1931 (Mirolubov, 1934), and these animals have been known as sources of human rabies since at least 1951. During the outbreak of 1979–80, active surveillance demonstrated rabies virus in  $13.5 \pm 4.6\%$  of raccoon dogs and in  $3.5 \pm 2.0\%$  of red foxes trapped randomly (Botvinkin et al., 1981). In an experimental study, susceptibility of raccoon dogs to indigenous rabies virus was significantly greater than susceptibility of foxes. During the clinical period, the animals were agitated and aggressive, and the virus was detected in their salivary glands more frequently than in the salivary glands of foxes (Botvinkin et al., 1983). These data provide an indirect suggestion that the raccoon dog might be the principal host of this rabies virus variant. However, about 80% of human rabies cases were caused by dogs, with raccoon dogs the next most important source. Only one human case was reported after a red fox bite (Savitsky et al., 1981; Yanovich, 2003).

In the Russian Far East, the rabies enzootic region is limited and fragmented. It lies in the wet lowlands along the Amur and Ussuri rivers. Most probably, a major part of the enzootic region is situated in the northern provinces of China. Unfor-

tunately, we had no samples from this area to make a comparison. The Komatsugawa virus, isolated from a dog in Tokyo shortly after the end of World War II (Ito et al., 1999), belonged to the same group.

Another lineage of group B (isolates 248c and 304c) originated from a local rabies focus situated in the highland steppes of East Siberia near the border with Mongolia (Transbaikalian region). During the first half of the 20th century, dogs and wolves were known as the main reservoirs and vectors of rabies virus in this area. Later, from 1950 to 1977, wolves were a source of 8.7% of human rabies cases and domestic animals (mainly dogs) were a source of the remaining 91.3%. Rabies in red fox was first diagnosed in 1961. From 1971 to 1977, rabid red foxes and steppe foxes were identified by veterinary laboratories almost annually, but foxes never caused a documented human case (Botvinkin et al., 1980). Both isolates, reported in this study, were found in 1977 as a result of active surveillance. Their Arctic-like properties were demonstrated in reactions with N-Mabs; both viruses reacted with N-MAb P-41 (Selimov et al., 1994). Rabies was not reported from this territory after 1984. It was not possible to discern whether this focus has been maintained. However, rabies is still occasionally reported from Mongolia (Angar, 2001).

Both group B lineages, as well as other

Arctic-like viruses originating from India and Pakistan, are separated from the northern area of Arctic rabies virus circulation by thousands of kilometers of allegedly rabies-free territory. It appears that these viruses originate from one progenitor, and current diversity could be the result of further independent evolution. However, it was not possible to make an appropriate age estimation because of lack of samples belonging to one lineage separated by a number of years.

Group C viruses formed a solid cluster with considerable bootstrap support. They were isolated in the huge steppe and forest-steppe territory of southeastern Europe, West Siberia, Kazakhstan, and Tuva. The last focus in Tuva is separated from the continuous western area of virus circulation by the Altay mountains. It appears to be a marginal zone of the rabies enzootic territory of the Mongolian highland steppes.

According to veterinary records and field surveillance data, the disease in the steppe zone of the FSU is maintained mainly by the red fox. The steppe fox participates in virus circulation concurrently with the red fox, particularly in territories of high population density (Sansizbaev, 1975; Malkov and Gribova, 1980; Kuzmin et al., 2001). We did not register any genetic distinctions between the red fox and steppe fox isolates.

In the area of group C circulation, the first wildlife rabies outbreak was reported among raccoon dogs, foxes, and wolves in 1945 in the region of the Volga River (Isakov, 1949). Since 1951, the red fox has been considered the main rabies reservoir (Amitrov, 1956; Nazarov, 1961). In 1949, a fox rabies outbreak was described in Kazakhstan (Sludsky, 1954). In West Siberia, fox rabies has been registered in Novosibirsk province since 1958 and in Altay province since 1961 (Vyazhevich, 1959; Kiryanov, 1962). Dogs (with sporadic cases caused by wolves) were the major source of human rabies in the first half of the 20th century, but foxes predominated as a

source of human rabies in the second half of the century (Selimov, 1978). Group D viruses originated from the western European part of Russia, including the junction of the Russian, Ukrainian, and Belorussian borders (so-called Polesie). This group retained an intermediate phylogenetic position between the lineages originating from other parts of Europe and group C, which was concordant with geographic distribution of the viruses. Particular antigenic patterns of these viruses were also suggested with N-MABs (Selimov et al., 1988). Red fox rabies, with involvement of other wildlife (the raccoon dog, badger (*Meles meles*), and sometimes small mustelids), predominated in this territory after World War II, and a high incidence of human rabies has been found there during the last few decades (Selimov, 1978; Cherkasskiy et al., 1995). We had only two human isolates originating from this region, one of them belonged to group D (RV239), but the second belonged to group C (RV241). Because of limited samples, we could not establish which virus lineage caused human disease more frequently.

Group E in our study was the same as the "northeastern Europe" group described earlier in Bourhy et al. (1999). The isolates from the Baltic region (northwestern part of Russia, Estonia, and Finland) reacted with N-MAB P-41 (Kulonen and Boldina, 1993; Selimov et al., 1994). Wildlife rabies predominated there after World War II (Selimov, 1978, 1998; Cherkasskiy et al., 1995). Because raccoon dogs were frequently involved in virus circulation in this region and because the viruses formed a separate lineage, the raccoon dog was proposed to be an intermediary host in the switch of rabies virus from dogs and wolves to foxes (Bourhy et al., 1999). However, there is no support for this statement in the epidemiologic surveillance data and historical veterinary records (Selimov, 1978, 1998; Cherkasskiy et al., 1995). The raccoon dog population density in the region is estimated at 3–20 times less than

that of the red fox population (Nasymovich and Isakov, 1985). It appears that the red fox is more affected in the Russian part of this area. However, raccoon dog cases predominated during the rabies outbreak in Finland in 1988–89 (Nyberg et al., 1992). Phylogenetically, this group was related to other European lineages, and there is no reason to consider it as specific to the raccoon dog.

The isolates originating from Georgia and Turkey tended to form another lineage that probably represented viruses circulating south of the Black Sea region (Johnson et al., 2003). One of the Georgian isolates (RV307) was joined to viruses originating from the Middle East. According to epidemiologic surveillance, dog rabies predominated in the Transcaucasian states of the FSU with only occasional incidence among wild canids (Selimov, 1978, 1998; Cherkasskiy et al., 1995). We have very few isolates from this territory to make appropriate comparisons. Different lineages of rabies virus appear to circulate in southeastern Europe and the Middle East (Bourhy et al., 1999; Johnson et al., 2003), although further investigations are needed. Because the viruses of group C were not identified south of the Caucasus Mountains, this mountain range could be a physical barrier separating these rabies virus populations.

All defined virus groups corresponded to certain geographic regions. Conversely, an association with host species was less obvious. The principal host might be identified only in Arctic regions (arctic fox) and in the Far East (raccoon dog). In other territories, rabies virus is apparently associated with foxes (red fox and steppe fox).

As has been suggested by others, the current European epizootic of fox rabies started in eastern Prussia or at the former Russia-Polish border during 1935–45 (Toma and Andral, 1977; Bourhy et al., 1999). Our data clearly demonstrate that viruses of groups B and C could not derive from that area. The distance from the western borders of Russia to the Volga del-

ta is about 1,500 km, and to Siberia and Kazakhstan about 3,000–3,500 km. The carrying capacity of biotopes and properties of fox populations in the western part of European Russia are similar to those described for central and western Europe. The speed of the epizootic front movement was estimated in Europe as 20–60 km per year (Toma and Andral, 1977; Pastoret and Brochier, 1999). With this velocity, the epizootic could not have reached the Volga River region by the time of the first outbreak there in 1945. In contrast, the estimated carrying capacity of Asian steppe biotopes appears limited. Fox populations here are mobile, and the animals seem to maintain rabies epizootics at a population density of 0.3–0.8 foxes per km<sup>2</sup> (Malkov and Gribanova, 1980). The speed of the epizootic front movement in Western Siberia was estimated as 160–513 km/yr (Rybak et al., 1992). There are no natural barriers to rabies progression, even during winter. Hence, the viruses of group C could spread along the steppe zone in Asia rapidly. It is not clear where the epizootic started. Additionally, small canids maintain viruses of group B, as well as other lineages of rabies virus in the Middle East (David et al., 2000; Johnson et al., 2003). Perhaps different populations of rabies virus independently switched to small canids in distinct areas of Eurasia during 1930–70.

Previous analyses of rabies virus sequences for evidence of positive selection have suggested that rabies virus evolves largely under purifying or neutral selection (Badrane and Tordo, 2001; Badrane et al., 2001; Holmes et al., 2002). Application of methods identical to those applied here for rabies virus isolates from different geographic regions suggested that positive selection was only occurring at a single codon in the ectodomain of the G gene (position 183). Both G gene data sets used in this study also selected position 183 as being under positive selection. Additionally, in this study, we found a further two codons to be under positive selection, cor-

responding to ~1% of the codons analyzed. Although this value is low, it remains in the range estimated for genes of other negative-strand RNA viruses (Woelk et al., 2002).

In nature, rabies virus exists in distinct lineages with little interaction of variants following division (Bourhy et al., 1999; Smith, 2002). We suggest that the detection of a greater number of codons under positive selection here might be a result of the restriction of this analysis to a defined subset of virus lineages, rather than an amalgamation of every available complete gene sequence (Holmes et al., 2002). In reality, processes of positive selection are likely to be lineage-dependent, and an assessment of positive selection across the entire rabies virus genotype could distort and dilute individual processes, such that they are no longer detectable. Ideally, the analysis here of Russian rabies virus isolates would have been further divided into individual variants. However, the number of sequences available limits adequate subdivision at this time.

The rabies virus glycoprotein is responsible for binding to host cell receptors and is a primary target of the immune response (Dietzschold et al., 1983). In contrast, the nucleoprotein is largely hidden from immune surveillance. The lack of detectable positive selection in the N gene concurs with the results of others (Holmes et al., 2002). All three of the G gene positive selection sites occur within the neurotoxin-like region (Lentz et al., 1984), a region thought to participate in host cell binding at neuromuscular junctions. These sites might be subject to adaptive evolution through host cell receptor binding, although, using the sequences from this study, we saw no obvious link between amino acid substitutions at these sites and phylogenetic placement. Although the majority of rabies virus replication occurs in neurons that are under relatively weak immune surveillance (Miller, 1999), it might be over simplistic to state that rabies virus evolves according to a neutral model (Bad-

rane and Tordo, 2001). Subtle positive selection at a handful of codons might be sufficient to ensure that limited immune pressure continues to force the rabies virus to adapt to changing host environments, especially in a diversity of small-bodied carnivores.

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