

Genetic similarity of Puumala viruses found in Finland and western Siberia and of the mitochondrial DNA of their rodent hosts suggests a common evolutionary origin

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Abstract

A total of 678 small mammals representing eight species were trapped in western Siberia in 1999–2000 and assayed for the presence of hantaviruses. Eighteen animals, all *Clethrionomys* species, were antigen positive by enzyme-linked immunosorbent assay (ELISA). Small and medium genome segments were recovered by RT-PCR from six samples from *Clethrionomys glareolus* and three from *Clethrionomys rufocanus*. Sequence comparison and phylogenetic analysis revealed that these hantaviruses were Puumala virus and were similar to hantavirus strains from Finland. To confirm these data, partial nucleotide sequences of the rodent hosts' cytochrome b genes were obtained, as well as several sequences from genes from rodents trapped at different localities of European Russia and western Siberia. The cytochrome b sequences of Siberian bank voles were similar to sequences of *C. glareolus*, trapped in Finland. These data suggest that the Puumala hantaviruses, as well as their rodent hosts, share a common evolutionary history. We propose that these rodents and viruses may be descendants of a population of bank voles that expanded northward from southern refugia during one of the interglacial periods.

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Keywords: Puumala virus; *Clethrionomys* spp.; Cytochrome b; Postglacial radiation; Western Siberia; Finland

1. Introduction

The *Hantavirus* genus (family *Bunyaviridae*) includes viruses that cause hemorrhagic fever with renal syndrome (HFRS). The hantavirus genome consists of a tripartite, single-strand, negative-sense RNA surrounded by a lipid membrane with two surface glycoproteins, G1 and G2. The large (L), medium (M) and small (S) genomic segments encode the viral polymerase, the glycoprotein precursor and

the nucleocapsid protein, respectively (Schmaljohn et al., 1986, 1987; Schmaljohn, 1990).

There are four serologically distinct groups of hantaviruses causing HFRS, represented by Hantaan (HTN), Dobrava (DOB), Seoul (SEO) and Puumala (PUU) viruses (Avsic-Zupanc et al., 1992; Lee et al., 1985; Schmaljohn et al., 1985; Sugiyama et al., 1987). They are isolated from the striped field mouse (*Apodemus agrarius*), yellow-necked field mouse (*Apodemus flavicollis*), rats (*Rattus norvegicus* and *R. rattus*) and bank voles (*Clethrionomys glareolus*), respectively.

HTN, DOB, SEO and PUU viruses cause the various clinical forms of HFRS characterized by fever, renal failure and, in severe cases, hemorrhagic manifestations. The mortality of HFRS varies from 0.2 to 10%, depending largely

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upon which hantavirus caused the infection (Schmaljohn and Hjelle, 1997).

In 1993 in the United States, the existence of another hantavirus-associated human disease, called hantavirus pulmonary syndrome (HPS), was reported. The predominant rodent host of the first HPS-causing virus identified, Sin Nombre virus (SN), is the deer mouse (*Peromyscus maniculatus*) (Childs et al., 1994). Since the discovery of SNV, numerous other hantaviruses able to cause HPS have been identified in North and South America (Enria et al., 2001; Rhodes et al., 2000) and hantaviruses not known to cause either HFRS or HPS have been found worldwide (Parrington and Kang, 1990; Plyusnin et al., 1994).

HFRS is endemic in Russia where approximately four cases per 100,000 people occur annually. However, the level of morbidity differs greatly in various parts of Russia. Thus, only a few cases of HFRS were detected in western Siberia over 20 years, although *Clethrionomys* species, the main carriers of PUU virus, are abundant in this territory, and findings of hantaviral antigens in rodents were reported repeatedly (Miasnikov et al., 1987, 1992). In contrast, Bashkortostan, which is close to the western Siberian part of central Russia, has the highest rate of HFRS morbidity in Russia (58.3 per 100,000 of population) (Tkachenko et al., 1998). The goal of this study was to determine the type of hantavirus circulating in western Siberia. This study also suggests new information with re-

gard to the evolutionary history of hantaviruses and their carriers.

2. Methods

2.1. Trapping and processing of rodents

Samples were obtained from rodents trapped in four locations of the Omsk region in a conifer forest (southern taiga). In addition, several samples from bank voles, *C. glareolus*, grey red-backed voles, *Clethrionomys rufocanus* and northern red-backed voles, *C. rutilus* from several other regions of Russia: Nizhniy Novgorod, Samara, Novosibirsk regions, Bashkortostan, Polar Ural and far east (Khabarovsk region) were used (Fig. 1). Animals were live-trapped (5–10 m between traps), humanely killed, species and sex identified, weighed, and placed in sterile individual plastic bags for transport to the laboratory. Inside the laboratory, the lungs were aseptically removed, and placed into individual cryovials. Cryovials were labeled with the rodent species, trapping site, date and the name of the technician. All tissues were stored at -70°C until tested.

In addition to the samples from trapped animals, 12 samples from *C. glareolus* (originating in Finland or Sweden) and two samples from *C. rufocanus* (from Kilpisjärvi, Finland) were obtained from University of Alaska Museum



Fig. 1. Map of Russia and Fennoscandia. Diamond-shape signs are locations where hantaviruses were found. Filled and open circles represent locations where *C. glareolus* of "glareolus" haplotype and "rutilus" haplotype, respectively, were trapped. Open squares are shown in locations where *C. rufocanus* were trapped. Arrows represent hypothetical migration routes.

collection and were similarly processed. The sample identifiers are: uam#22039, 30028, 30046, 30048, 24441, 24444, 24446, 24469–24471, 24473–24476 (Table 1).

2.2. Antigen detection

Hantaviral antigens were detected by using enzyme-linked immunosorbent assay (ELISA)-antigen capture as described elsewhere (Verhagen et al., 1986). Briefly, 10% lung suspensions were added to microtiter plates containing affinity-purified anti-PUU virus IgG from convalescent sera, with immunofluorescent antibody (IFA) titers not less than 16,000. The plates were incubated at 37 °C for 2 h, washed and anti-PUU virus IgG–peroxidase conjugate was added followed by incubation for 1 h at 37 °C. Color-developing substrate was added and OD at 492 nm was measured in an ELISA reader. Positive samples were subjected to RT-PCR.

2.3. Preparation of viral RNA, rodent mitochondrial DNA (mtDNA) and nuclear DNA (ncDNA)

Total RNA was extracted from lung suspensions of samples identified by ELISA as having hantaviral antigens. DNA from lung, brain, heart and skin was extracted by using TRIzol reagent (Gibco BRL). ncDNA and mtDNA were isolated using a DNeasy Tissue kit (QIAGEN) according to the manufacturer's protocol.

2.4. RT-PCR and sequencing

RT-PCR reactions were carried out using random hexamer or genus-reactive primers (Table 2) with reagents purchased from Gibco BRL and from Perkin-Elmer. PCR products were gel-purified and directly sequenced on either an ABI 377 Genetic Analyzer (PE Applied Biosystems), or manually, using a Sequenase, Version 2.0 sequence kit (US Biochemical), according to the manufacturer's instructions.

2.5. Random amplification of polymorphic DNA (RAPD)

Working aliquots of DNA samples were prepared by bringing them to a final concentration of 10 ng/μl in TE buffer (10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA). Forty (40) random amplification of polymorphic DNA (RAPD) primers (Operon Technologies), OPAD-01–OPAD-20 and OPAE-01–OPAE-20, were screened for reproducibility of bands, and three primers (OPAE-01, OPAE-18, and OPAD-14) were chosen for the final analysis. Amplification reactions were performed in volumes of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl₂, 100 μM of each dNTP, 5 pmol of RAPD primer, 50 ng of genomic DNA and 0.5 U of Taq DNA polymerase. Amplifications were performed under these conditions: 1 min at

94 °C, 1 min at 33 °C, and 2 min at 72 °C, 45 cycles. Amplification products were resolved by electrophoresis in 1.4% agarose and detected by staining with ethidium bromide.

2.6. Phylogenetic analysis

Multiple sequence alignment was performed with the Clustal W program (Thompson et al., 1997). Phylogenetic analysis for viral sequences of S- and M-genome segments and for rodents' cytochrome b sequences was performed by maximum-parsimony (MP), neighbor-joining (NJ) and maximum-likelihood (ML) methods. Phylogenetic analysis was conducted by using the programs PAUP, Version 4.0β10 (Swofford, 2000), MEGA, Version 2.0 (Kumar et al., 2000), and TREE-PUZZLE, Version 4.0.2 (Strimmer and von Haeseler, 1999), for MP, NJ and ML phylogenetic methods, respectively.

MP nucleotide sequences of the S-segment and partial M-segment was analyzed with a heuristic search using stepwise addition and performing tree-bisection-reconnection branch swapping. Support for each clade was assessed by bootstrap analysis from 1000 replicates. Unweighted MP analysis was carried out in all cases unless relationships were not resolved by any other methods. Then, the characters were a posteriori weighted depending on rescaled consistency index. NJ analysis was carried out with distance matrices calculated with the Kimura two-parameter model and the relative support for the nodes was assessed by the bootstrap test. ML analysis was performed with the Tamura-Nei model using quartet puzzling.

For comparison, existing nucleotide sequences of hantaviral genomes and rodent cytochrome b were obtained from GenBank. The sequences obtained in this study have been deposited in GenBank and the accession numbers listed in Table 1.

2.7. Maximum-likelihood estimation of migration rates and effective population sizes

Maximum-likelihood estimates for migration rates and effective population sizes were obtained using computer program "Migrate", Version 1.6.7 (Beerli and Felsenstein, 2001) for subpopulations from the Omsk region (17 sequences), Finland (7 sequences), Nizhniy Novgorod region (14 sequences), and Samara region (11 sequences). Input files consisted of interleaved aligned sequences of cytochrome b (590 nt long).

A transition/transversion ratio of 16.2 was estimated from the data set using the TREE-PUZZLE program. Markov chain initial settings were as follows: number of short chains—10 (trees sampled—10⁴, trees recorded—500), number of long chains—3 (trees sampled—10⁵, trees recorded—5000). Because in a substantial number of runs the program returned improbably high Θ values, the number of short chains was changed to 40 and runs were made with adaptive heating (temperatures = 1, 2, 4, 8).

Table 1
Virus and rodent samples used in the study

	Sample	Geographic location/year of collection	Host	S-segment assn #	M-segment assn #	Host cytochrome b assn #	D-loop assn #
1	CG144	Omsk region/1999–2000	<i>C. glareolus</i>	AF367064	AF367055/G2	AF367079	
2	CG168	Omsk region/1999–2000	<i>C. glareolus</i>	AF367065	AF367056/G2	AF429781	
3	CG182	Omsk region/1999–2000	<i>C. glareolus</i>			AF429782	AY133306
4	CG191	Omsk region/1999–2000	<i>C. glareolus</i>			AF429783	
5	CG193	Omsk region/1999–2000	<i>C. glareolus</i>			AF429784	AY133307
6	CG196	Omsk region/1999–2000	<i>C. glareolus</i>			AF429785	
7	CG206	Omsk region/1999–2000	<i>C. glareolus</i>			AF429786	AY133308
8	CG215	Omsk region/1999–2000	<i>C. glareolus</i>	AF367066	AF442615/G1G2	AF429787	AY133309
9	CG216	Omsk region/1999–2000	<i>C. glareolus</i>			AF429788	AY133310
10	CG222	Omsk region/1999–2000	<i>C. glareolus</i>	AF367067	AF442616/G1G2	AF367074	
11	CG315	Omsk region/1999–2000	<i>C. glareolus</i>	AF367068	AF367059/G2	AF429789	AY133312
12	CG463	Omsk region/1999–2000	<i>C. glareolus</i>		AF367060/G2	AF429790	AY133315
13	CRF161	Omsk region/1999–2000	<i>C. rufocanus</i>	AF367069	AF367061/G1G2	AF367072	AY133316
14	CRF179	Omsk region/1999–2000	<i>C. rufocanus</i>			AF429791	AY133317
15	CRF308	Omsk region/1999–2000	<i>C. rufocanus</i>	AF367070	AF442617/G1G2	AF429792	AY133319
16	CRF333	Omsk region/1999–2000	<i>C. rufocanus</i>			AF429793	AY133320
17	CRF366	Omsk region/1999–2000	<i>C. rufocanus</i>	AF367071	AF367063/G2	AF367078	
18	CG/AF#1946	Finland, Sotkamo/1986	<i>C. glareolus</i>			AF429812	AY133325
19	CG/AF#3306	Finland, Sotkamo/1986	<i>C. glareolus</i>			AF429813	AY133326
20	CG/uam24469	Finland, Pieksämäki/1982	<i>C. glareolus</i>				AY185807
21	CG/uam24470	Finland, Pieksämäki/1982	<i>C. glareolus</i>				AY185809
22	CG/uam24471	Finland, Pieksämäki/1982	<i>C. glareolus</i>			AY185799	AY185808
23	CG/uam24473	Finland, Pieksämäki/1982	<i>C. glareolus</i>				AY185802
24	CG/uam24474	Finland, Puhos/1982	<i>C. glareolus</i>			AY185796	AY185801
25	CG/uam24475	Finland, Pieksämäki/1982	<i>C. glareolus</i>			AY185797	AY185803
26	CG/uam24476	Finland, Pieksämäki/1982	<i>C. glareolus</i>			AY185798	AY185804
27	CRF/AF#3143	Finland, Kilpisjärvi/1987	<i>C. rufocanus</i>			AF429814	AY133327
28	CRF/AF#3145	Finland, Kilpisjärvi/1987	<i>C. rufocanus</i>			AF429815	AY133328
29	CG/uam24441	Sweden, Båtskärsnäs/1982	<i>C. glareolus</i>			AY185800	AY185805
30	CG/uam24444	Sweden, Båtskärsnäs/1982	<i>C. glareolus</i>				AY185810
31	CG/uam24446	Sweden, Båtskärsnäs/1982	<i>C. glareolus</i>				AY185806
32	CG3B	Bashkortostan/2001	<i>C. glareolus</i>			AF429794	
33	CG17B	Bashkortostan/2001	<i>C. glareolus</i>	AF442613	AF442614		
34	CG28B	Bashkortostan/2001	<i>C. glareolus</i>			AF429795	
35	CG29B	Bashkortostan/2001	<i>C. glareolus</i>			AF429796	
36	CG40B	Bashkortostan/2001	<i>C. glareolus</i>			AF429797	
37	CG9s	Samara region/2001	<i>C. glareolus</i>			AY062900	
38	CG10s	Samara region/2001	<i>C. glareolus</i>			AY062901	
39	CG11s	Samara region/2001	<i>C. glareolus</i>			AY062902	
40	CG12s	Samara region/2001	<i>C. glareolus</i>			AY062903	
41	CG13s	Samara region/2001	<i>C. glareolus</i>			AY062904	
42	CG14s	Samara region/2001	<i>C. glareolus</i>			AY062905	
43	CG15s	Samara region/2001	<i>C. glareolus</i>			AY062906	
44	CG16s	Samara region/2001	<i>C. glareolus</i>			AY062907	
45	CG28s	Samara region/1998	<i>C. glareolus</i>	AF411448		AF367082	
46	CG142s	Samara region/1998	<i>C. glareolus</i>	AF411447		AF367080	
47	CG230s	Samara region/1998	<i>C. glareolus</i>	AF411449		AF367081	
48	CG18NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429798	
49	CG24NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429799	
50	CG27NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429800	
51	CG33NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429801	
52	CG34NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429802	
53	CG35NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429803	
54	CG36NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF367083	AY133323
55	CG37NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429804	
56	CG40NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429805	
57	CG42NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429806	
58	CG44NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429807	
59	CG45NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429808	

Table 1 (Continued)

	Sample	Geographic location/year of collection	Host	S-segment assn #	M-segment assn #	Host cytochrome b assn #	D-loop assn #
60	CG56NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF367084	AY133324
61	CG58NN	Nizhniy Novgorod region/1996	<i>C. glareolus</i>			AF429809	
62	CG12H	Novosibirsk region/2000	<i>C. glareolus</i>			AF367075	AY133303
63	CG14H	Novosibirsk region/2000	<i>C. glareolus</i>			AF429810	AY133304
64	CG15H	Novosibirsk region/2000	<i>C. glareolus</i>			AF367076	
65	CG16H	Novosibirsk region/2000	<i>C. glareolus</i>			AF429811	AY133305
66	CRF443	Polar Ural, Yanganope/2000	<i>C. rufocanus</i>			AF367072	AY133321
67	CRF890	Khabarovsk region/2000	<i>C. glareolus</i>			AF429816	
68	CRT481	Omsk region/1999	<i>C. rutilus</i>			AF367077	

3. Results

3.1. Detection of viral RNA in rodent samples

A total of 678 rodents representing eight species were trapped. They included (total/ELISA-positive/PCR-positive): *C. glareolus*—161 (23.7%)/9/6, *C. rufocanus*—109 (16.1%)/6/3, *C. rutilus*—306 (45.1%)/3/0, *A. agrarius*—26 (3.8%), *Microtus arvalis*—2 (0.3%), *M. agrestis*—43 (6.3%), *M. oeconomus*—30 (4.4%) and *Micromys minutus*—1 (0.1%).

Nine of the lung suspensions from the Omsk samples (CG144, CG315, CG463, CG168, CG215, CG222, CRF308, CRF366 and CRF161) were found to have viral RNA when M-segment primers were used for PCR. PCR performed with S-segment primers yielded products of the expected size (approximately 1700 nucleotides) from all Omsk samples, except CG463. All samples from Bashkortostan yielded PCR products with both the S- and M-segment primers, however only one sample (CG17B) was sequenced. For samples from the Samara, Nizhniy Novgorod and Novosibirsk regions, we only performed PCR using the S-segment primers. Fragments having the expected size were recovered from each of three samples from the Samara region (CG28, CG142, and

CG230) and from one sample from the Nizhniy Novgorod region (CG38).

3.2. Hantaviral sequence comparisons

We determined the nucleotide sequences of the carboxy terminal coding regions of the G2 genes (865 nucleotides) of all of the PCR-positive Omsk samples, and the entire M-segment sequences (with the exception of terminal 5' and 3' stretches complementary to primers) of four of the samples (CG215, CG222, CRF161 and CRF308). Comparing the M-segment regions amplified from the Omsk samples revealed nucleotide and amino acid sequence identities ranging from 99.2 to 100 and 99.2 to 100%, respectively. The S-segment region amplified from the Omsk samples included the entire nucleocapsid ORF (1302 nucleotides) and most of the 3' non-coding region (434 nucleotides). The coding regions of these samples had nucleotide and deduced amino acid identities of 99.2–100 and 99.3–100%, respectively. The 3' non-coding regions were 98.4–99.8% identical. There was a four-nucleotide insertion (ATAT) in the non-coding region of sample CRF366.

The Omsk samples had specific nucleocapsid (N) protein amino acid “signatures” (Sironen et al., 2001) that

Table 2
Primers used in the study

	Name	Sequence	Polarity	Amplified region	Position	Reference sequence
1	PUUsN	GCTACTACGARGAMRACTGGA	Pos	Hantavirus S-segment	22–42	X61035
2	PUUs3'	GAAATCAGCATGTTGAGGTAGTA	Neg	Hantavirus S-segment	1734–1756	X61035
3	mG2-F	TTAGAAAGAAATGTGCATTTGC	Pos	Hantavirus M-segment	2742–2763	M29979
4	PUUm5'	TAGTAGTAGACTCCGCAAGAAG	Pos	Hantavirus M-segment	1–22	M29979
5	PUUm3'	TAGTAGTAGACTCCGCAAGAAC	Neg	Hantavirus M-segment	3661–3682	M29979
6	CB-F	ATGACARTC ATCCGAAARAAACAC	Pos	Cytochrome b	1–24	AF119272
7	CB-R	TTARTCTAGGTCYAKRATGTYGTTTTC	Neg	Cytochrome b	1117–1143	AF119272
8	dL2OP-F	ACATTCTATTTAACTACTTCT	Pos	D-loop	73–94	Y07543
9	dL2OP-R	GAAGTAGGAACCAGATGTCTG	Neg	D-loop	453–473	Y07543
10	SRYhmg-F	GTCAAGCGCCCCATGAATGCA	Pos	Sex-determining region (HMG-box), chr Y	99–119	L29543
11	SRYhmg-R	AGTTTGGGTATTTCTCTCTGT	Neg	Sex-determining region (HMG-box), chr Y	280–300	L29543
12	brca1-F	AAGACAGAATGAATGTAGAAAAGGCTG	Pos	breast cancer susceptibility gene (<i>BRCA1</i>)	105–122	AF332048
13	brca1-R	TTTCTTGATAARGTCCTCAGG	Neg	breast cancer susceptibility gene (<i>BRCA1</i>)	756–776	AF332048

Table 3

Comparison of the nucleotide and amino acid sequences of CRF161/Omsk-2000 with other Puumala-type hantaviruses and prototype hantaviruses of other types (% identity)

Country	Strain	S ^a	N	M ^b	G2 ^c
Russia	CG-1820	85.6	95.8	82.3	94.5
Russia	P360	85.3	95.4	82.6	94.9
Russia	Udm/894Cg/91	85.9	96.3	83.2	95.8
Russia	Kazan	85.4	96.3	83.2	95.8
Russia	CG-Kolodozero	87.2	97.2	nd ^d	nd
Finland	Sotkamo	88.5	97.9	82.2	91.1
Finland	PUU/1324Cg/79	89.2	98.2	nd	nd
Finland	Evo/12Cg/93	87.8	97.5	nd	nd
Finland	Virrat/25Cg/95	87.9	97.7	nd	nd
Denmark	CG-Fyn	84.0	96.1	nd	nd
France	CG13891	84.0	95.8	80.2	93.2
Germany	Erft	84.0	95.0	80.7	94.9
Sweden	Vindeln/L20Cg	84.5	97.0	80.7	90.3
Sweden	Mellansel/cg47/94	83.6	96.3	nd	nd
Sweden	Hundberget/cg36/94	84.1	96.8	nd	nd
Sweden	Solleftea/cg3/95	85.4	97.7	nd	nd
Norway	Eidsvoll/1124v	84.7	96.1	81.1	85.5
Japan	Kamiiso	82.9	96.1	77.3	91.5
	TOP/Ls136V	76.2	86.4	72.8	79.3
	KBR/mf43	75.6	86.6	73.0	81.0
	TUL/Tula/53ma/87	74.2	79.9	73.1	79.3
	SN/CC107	68.0	70.9	69.2	71.1
	HTN/76–118	61.4	60.3	61.4	57.7
	DOB/Dobrava/3970/87	61.9	60.7	60.8	56.6
	SEO/SR-11	61.4	61.4	61.7	59.9

^a Coding part of S-segment.

^b Coding part of M-segment.

^c aa913–1148 (corresponding to amino acid sequence of G1–G2 precursor of PUU strain Sotkamo).

^d Not determined.

were similar, although not identical, to those reported for Finnish strains of hantaviruses. While M₂₆₂ and D₃₀₄ were present in all of our Omsk N proteins, F₃₈₈ was replaced by Y₃₈₈ in all sequences. As for those “signatures” that are shared by Finnish and Russian lineages, Y₆₁ was conserved in all Omsk sequences, while V₃₄ was substituted by M₃₄. Unexpectedly, the S-segment sequences of the Omsk samples were closer to those of Finnish strains (87.8–89.2%) than to previously characterized Russian strains (85.3–87.2% identity) (Table 3). However, the M-segment sequences were closer to the Russian strains, Udmurtia and Kazan (83.2%), than to the Finnish strain Sotkamo (82.2%).

The S-segment nucleotide sequences of samples from the Samara and Nizhny Novgorod regions were closer to each other (92–93% identity) than to samples from the Omsk region (81–84% identity). We determined the complete S- and M-segments sequences for the sample CG17/Bashkortostan-2001 (CG17B) and found them to be 99.3 and 99.1%, respectively, identical to those of a virus (CG1820) from the same region characterized earlier (Giebel et al., 1989; Stohwasser et al., 1990).

3.3. Rodents' sequence comparisons

There is strong evidence that hantaviruses have co-evolved with their rodent hosts (Plyusnin and Morzunov, 2001); consequently, we were interested in determining if the close identity of the Omsk hantaviral sequences and the Finnish sequences that we observed was reflected in a close genetic similarity of their rodent hosts. To assess the genetic similarities of the rodents, we chose two regions of rodents' mitochondrial DNA (mtDNA), cytochrome b encoding region, and D-loop, part of the control region, two genetic markers, commonly used in evolutionary studies (Johns and Avise, 1998; Sunnucks, 2000). We extracted DNA from rodent's tissues and PCR-amplified a 1060 nt fragment of the cytochrome b gene of numerous rodent samples from Russia, Finland and Sweden. Comparing the nucleotide sequences of the fragments revealed two major mitochondrial haplotypes, “glareolus” and “rutilus”, co-existent over the territory covered by the study.

Rodents from Samara, Nizhny Novgorod, Omsk, and part of Novosibirsk (west bank of river Ob) regions, as well as from Finland, belonged to the “glareolus” haplotype and were very close to each other with overall identities of 98.6–100%. Among them we were able to find several identical haplotypes, mostly among animals trapped in the same locations (CG18NN, CG24NN, CG27NN, CG34NN, CG44NN and CG42NN and CG56NN—from the Nizhny Novgorod region; CG9S, CG11S and CG12S, CG14S and CG230S—from the Samara region; CG193, CG215 and CG168, CRF366, and CG182, CG467, CRF308—from the Omsk region). However, identical haplotypes were also found in different locations—one in the Omsk and Nizhny Novgorod regions, and in Finland (specimens CRF161, CG216, CG315, CG58NN and CG/uam24476), and another in the Omsk region and Finland (CG144 and CG/AF3133).

The “rutilus” haplotype was found in animals from Bashkortostan and the Novosibirsk region (east bank of river Ob), as well as in Sweden (vicinity of Båtskärsnäs), with 98.4–100% sequence identities. Of these, only two were found to be identical, those of the Bashkirian samples, CG28B and CG29B.

Comparing all of the “glareolus” to the “rutilus” haplotypes revealed identities from 92.1 to 92.7%. The cytochrome b gene sequence that we determined for *C. rutilus* (CRT481), trapped at the same locality as all bank voles from the Omsk region samples, was similar to other sequences of grey red-backed voles available in GenBank. All sequences of “rutilus” haplotype bank voles from Bashkortostan, the Novosibirsk region and Sweden were close (within 98.4–99.9% of nucleotide sequence similarity) to sequences of CRT481.

It is noteworthy that sequences of the cytochrome b genes from *C. rufocanus* samples trapped in the Omsk region were almost identical to sequences from *C. glareolus* and, in contrast, substantially differed from cytochrome b of *C. rufocanus* (CRF443) from the Polar Ural, as well as from other

cytochrome b gene sequences from grey red-backed voles that are available in GenBank.

To further examine the genetic relationships of the rodents, we also PCR-amplified and sequenced a partial D-loop region of mitochondrial DNA of selected samples from Omsk, Novosibirsk and Nizhniy Novgorod regions; Finland, Sweden and also from the sample CRF443 (Polar Ural) (Table 1).

As with the cytochrome b gene fragments, we found identical D-loop sequences among samples originating from the same region, as well as among those from different localities. From the Omsk region, samples CG182, CRF308, CRF468; samples CG216, CG315, CG193, CG221; samples CG325, CRF333; and samples CRF161, CRF187 were identical within the sequenced region. The sequence of CG467 from the Omsk region was identical to CG36NN from the Nizhniy Novgorod region. The largest group of identical samples included CG206, CRF179, CG324, CG215 from the Omsk region; CG16H from Novosibirsk region; CG56NN from the Omsk region; and samples CG/AF#1946, CG/AF#3306, CG/uam24469, CG/uam24470, CG/uam24473, CG/uam24475 and CG/uam24476 from Finland.

Also like the cytochrome b sequences, D-loop sequences separated the rodents into two major groups “*glareolus*” and “*rutilus*.” The first group included all samples from Finland, the Nizhniy Novgorod and Omsk regions, as well as the sample from Novosibirsk region (CG16H). Within this group, we found nucleotide identities from 99.2 to 100%. The second group comprised of two samples from Sweden (CG/uam24441 and CG/uam24446) and two samples from Novosibirsk region (CG12H and CG14H) were 98.0–99.2% identical. One sample from Sweden (CG/uam24444) was not included in either group as it had pronounced differences when compared to sequences belonging to either group (97.2–97.7% identity to the samples from “*glareolus*” group and 93.2–93.8% identity to the samples from the “*rutilus*” group).

3.4. Phylogenetic analysis

Phylogenetic analysis of partial S-segment sequences (630 nt) revealed well-supported monophyly of the Omsk and Finnish PUU virus strains with bootstrap values between 88 and 96% (Fig. 2). Notably, viruses from the Nizhniy Novgorod and Samara regions clustered with other Russian strains within a well-supported branch (97%). Excluding these samples and including sequences of the entire N-coding region of the S-segments of the rest of the samples did not change the branching pattern or bootstrap values.

Phylogenetic analysis of the partial M-genome segment differed from that of the strains from European Russia (Udmurtia, Kazan, CG1820, K27, CG17/Bashkortostan). Omsk strains and the Finnish strain (Sotkamo) form a polytomy, consistently observed by unweighted NJ, MP and ML methods. Polytomy was resolved only using weighted MP analy-

sis by a successive weighting of characters based on rescaled consistency index and the best fit of the characters over all equally parsimonious trees. The successive weighting procedure was iterated until no change in tree length occurred. On the tree generated after analysis of the G2 encoding portion of the M-segment, the Omsk samples (CRF161, CRF308, CG215 and CG222) clustered together with PUU virus strains from European Russia, with the Sotkamo strain being an outgroup to them and the Omsk samples (Fig. 3a). In contrast, on the tree resulting from phylogenetic analysis of the entire M-segments, the Omsk samples positioned as an outgroup to both the European Russian hantaviruses and to the PUU virus strain Sotkamo from Finland (Fig. 3b).

In addition to viral sequences, we attempted to analyze the phylogenetic relationships of the partial cytochrome b gene sequences or D-loop sequences of rodents from different locations. The sequence from *C. rufocanus* from the Polar Ural (CRF443) was used as an outgroup. All attempts using common phylogenetic methods (NJ, MP, ML) failed, most likely because of extremely high degree of similarity between samples. Only two major clades, “*rufocanus*” and “*rutilus*” were well supported (>70%).

3.5. Estimation of effective population sizes and migration rates

The presence of identical mitochondrial haplotypes in animals from different locations, including the Omsk and Nizhniy Novgorod regions, and Finland, suggests a gene flow between subpopulations of those locations in the past or at present. We attempted to estimate a direction of gene flow by using a Markov chain Monte Carlo approach (computer program “Migrate”). The program calculates maximum-likelihood of a genealogy given the population parameters, such as population size and migration rates. Parameters obtained in the program were Θ (Θ is equal to $2 \times$ effective population size $\times$ mutation rate/site/generation) and Nm (effective population size $\times$ immigration rate/generation). Example estimates presented in Table 4 suggest that there is a gene flow directed toward the Finnish subpopulation from the Omsk region subpopulation. Other consistently observed values were gene flows from Nizhniy Novgorod region to Omsk and Samara regions. All other values in the migration matrix table were close to zero.

3.6. Nuclear DNA analysis

As another means to detect differences among the *C. rufocanus* and *C. glareolus* samples from the Omsk region (which were otherwise similar when mtDNA was analyzed), we examined two genes from the nuclear genome: sex-determining region, HMG-box (*Sry-HMG*) of chromosome Y and breast cancer susceptibility gene (*BRCA1*).

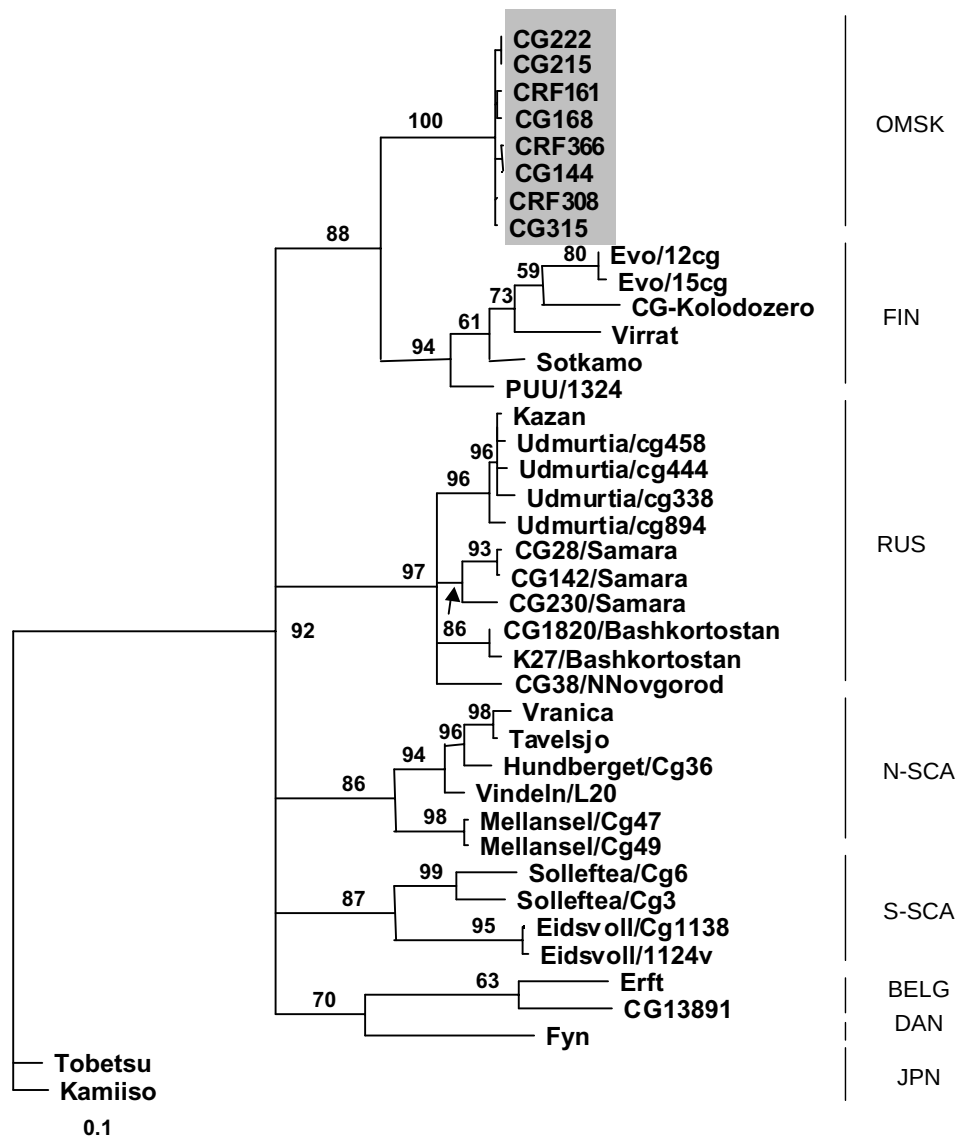


Fig. 2. Phylogenetic analysis based on partial sequences of the S-segment (630 nt), generated by maximum-likelihood method. Bootstrap values are expressed as percentages of the total number of replicates (10,000). PUU viruses originating from the Omsk region are indicated by shading. The S-segment sequences included PUU strains: Sotkamo (X61035), PUU/1324Cg/79 (Z46942), Evo/12Cg/93 (Z30702), Evo/15Cg/93 (Z30705), Virrat/25Cg/95 (Z69985), CG13891 (U22423), PUU/Cg-Erft (AJ238779), Fyn (AJ238791), Vindel/L-20 (Z48586), Mellansel/Cg47/94 (AJ223374), Mellansel/Cg49/94 (AJ223375), Hunberget/Cg36/94 (AJ223371), Solleftea/Cg3/95 (AJ223376), Solleftea/Cg6/95 (AJ223377), Tavelsjo/Cg81/94 (AJ223380), Eidsvoll/1124v (AJ223368), Eidsvoll/Cg1138/87 (AJ223369), Vranica (U14137), CG-Kolodozero (AJ238789), CG1820/Ufa-83 (M32750), K27/Ufa-85 (L08804) Udmurtia/894Cg/91 (Z21497), Udmurtia/338Cg/92 (Z30708), Udmurtia/444Cg/88 (Z30706), Udmurtia/458Cg/88 (Z30707), Kazan (Z84204), Tobetsu-60Cr-93 (AB010731) Kamiiso-8Cr-95 (AB010730). Genetic lineages indicated by abbreviations on the right.

Table 4
Estimation of Θ and ΘM for subpopulation from four localities

Population (x)	Number of sequences	Log likelihood	Θ ($2N_e\mu$) ^a	ΘM ^b			
				1 → x	2 → x	3 → x	4 → x
1 Nizhniy Novgorod r.	14	4.853	0.01099	–	1.98×10^{-12}	1.18×10^{-13}	2.78×10^{-11}
2 Samara r.	11	4.853	0.00163	0.68	–	3.55×10^{-14}	6.32×10^{-11}
3 Finland	7	4.853	0.00042	1.66×10^{-10}	2.23×10^{-13}	–	19.23
4 Omsk r.	17	4.853	0.00748	3.94	7.65×10^{-14}	1.27×10^{-13}	–

^a N_e : effective population size; μ : mutation rate per site per generation.

^b M : migration rate per generation ($M = m_i/\mu$).

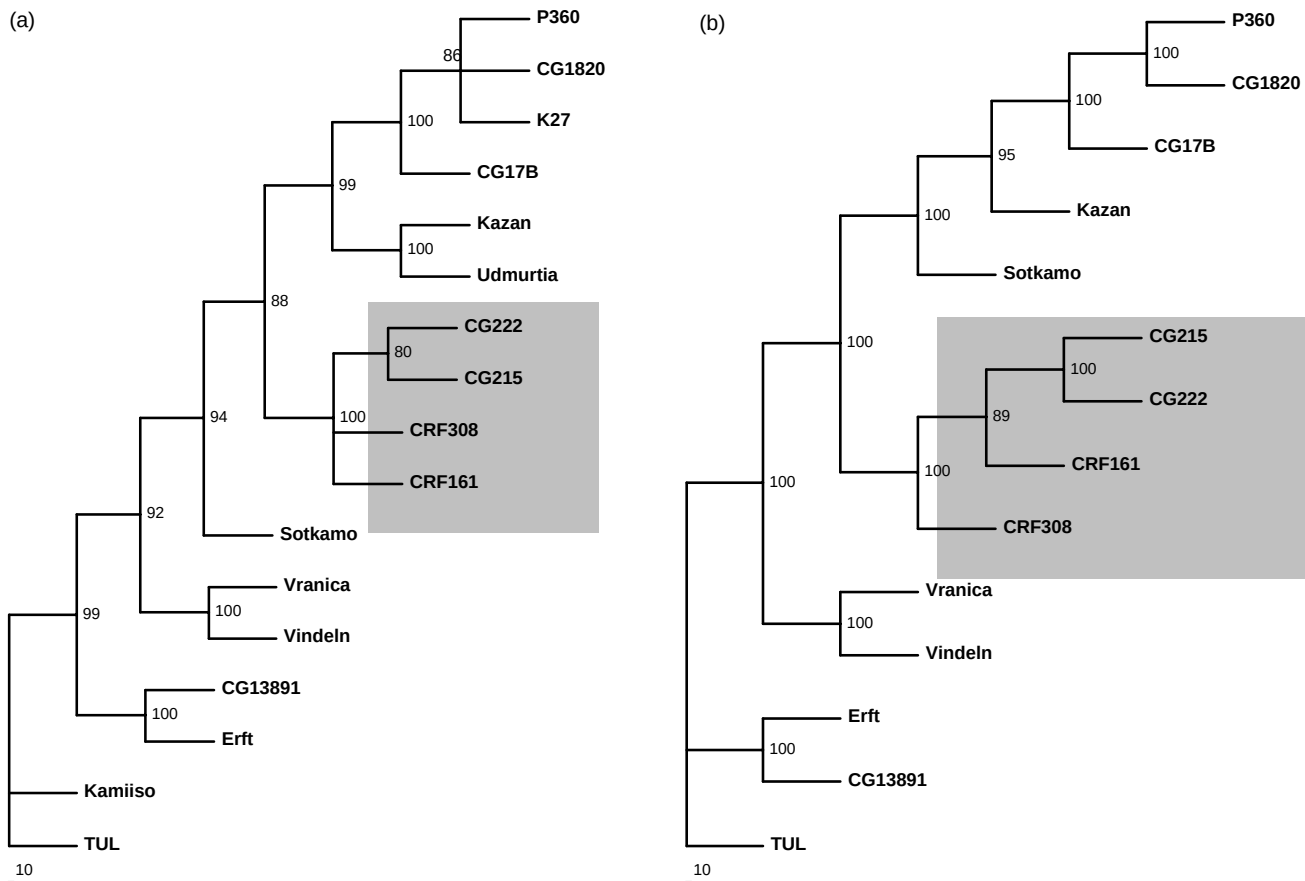


Fig. 3. Phylogenetic analysis based on (a) partial sequences of the M-segment (885 nt) and (b) sequences of the entire coding region of the M-segment. Tree (a) is a consensus tree from the three most parsimonious trees generated by maximum-parsimony method (1000 bootstrap replicates). Trees were constructed using successive weighting based on a rescaled consistency index. PUU viruses originating from the Omsk region are indicated by shading. The M-segment sequences included strains: Sotkamo (X61034), CG13891 (U22418), CG1820/Ufa-83 (M29979), K27/Ufa-85 (L08754), Vindeln/L20Cg (Z49214), Vranica (U14136), Udmurtia/894Cg/91 (Z21509), Kazan (Z84205), Eidsvoll/1124v (AJ223367), Kamiiso/8Cr/95 (AB011631), PUU-Erft (AJ238778), Tula/Moravia/5286 Ma/94 (Z66538).

For the analysis of Sry-HMG of chromosome Y, which is known to exhibit rapid amino acid divergence among species and little to no variation within species (Tucker and Lundrigan, 1995), samples from male voles were examined (CG12H, CG167, CG215, CRF161, CRF302, CRF443 and CRF/AF3143). The sequences obtained were aligned against the Sry-HMG sequence of *C. glareolus* from Spain available in GenBank (AJ132883). Within the 158 nt region analyzed, the Omsk sequences displayed three nucleotide differences, CRF443 and CRF/AF3143—two nucleotide differences, and CG12H—one nucleotide difference from AJ132883. All sequences from the Omsk samples (both *C. glareolus* and *C. rufocanus*) were identical.

The *BRCA1* gene was selected for analysis because it had given robust resolution of relationships at varying levels of divergence in previous studies (Adkins et al., 2001) and was easily amplified. *C. glareolus* and *C. rufocanus* samples from Omsk (CG/AF1946, CG191, CG193, CG324, CRF179, CRF302 and CRF443) were randomly selected and 497 nt fragments of their *BRCA1* genes were compared. Only two

of the samples differed: CRF443 and CG/AF1946, which had 2.6 or 0.2% nucleotide differences as compared to the other Omsk samples.

3.7. RAPD

To further analyze the Omsk samples, we performed RAPD analysis (Williams et al., 1990) using primers OPAE-01, OPAE-18 and OPAD-14. The first two primers gave identical polymorphism patterns, although some correspondent bands had different intensities for *C. rufocanus* and *C. glareolus* samples (not shown). When amplified using OPAD-14 primer, a pattern of polymorphism was similar; however, all samples from *C. rufocanus* demonstrated a weak band approximately 900 bp long, whereas such a band was absent in the *C. glareolus* amplification pattern (Fig. 4). Notably, the amplification pattern of *C. rufocanus* samples from the Omsk region, substantially differed from the pattern of other samples from gray red-backed voles, CRF3143, CRF3145, CRF443 and CRF718,

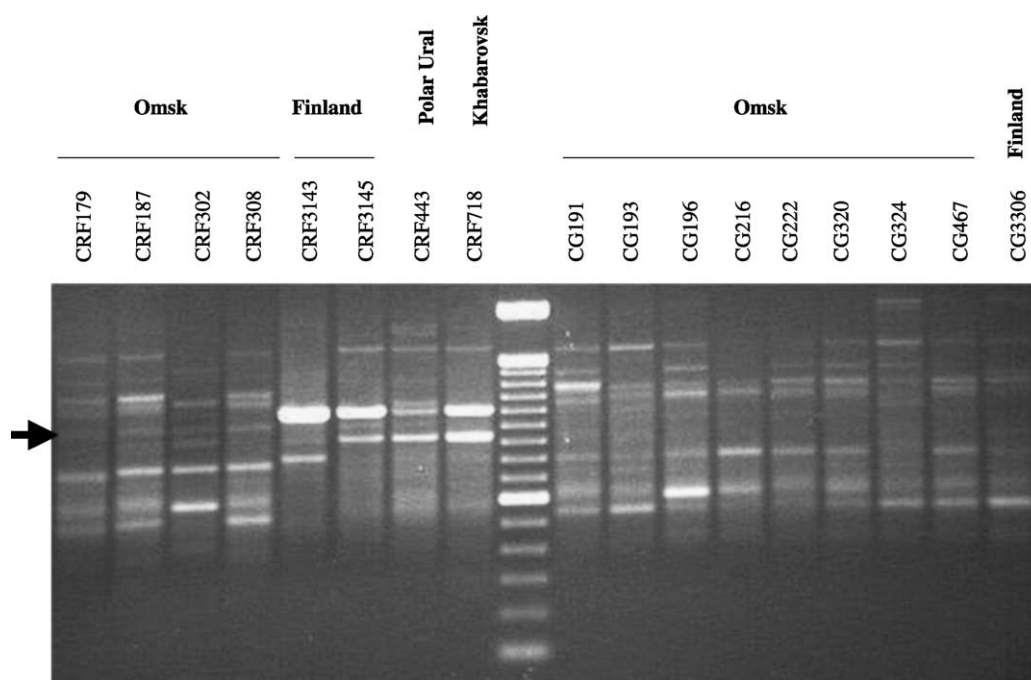


Fig. 4. Random amplification of polymorphic DNA (RAPD) using OPAD-14 primer (Operon Technologies) of *C. rufocanus* and *C. glareolus* samples from different geographic localities. Molecular weight marker was 100 bp DNA ladder (Gibco BRL). An arrow indicates a band (~900 bp) that is characteristic only for samples of *C. rufocanus* from the Omsk region.

collected in Finland, Polar Ural and far eastern Russia, respectively.

4. Discussion

Until recently, the diversity of hantaviruses to the east of the Ural mountains in Russia was unknown. Even their presence in Siberia was questioned, as there were no confirmed cases of human disease associated with hantaviruses in this area for over 20 years. A study (Vapalahti et al., 1999) of Siberian lemmings in the arctic tundra (Yamal peninsula, western Siberia) established the existence of a novel hantavirus, Topografov virus. However, because the lemmings' habitat is generally limited to the tundra, which is almost uninhabited, it is highly unlikely that lemmings make a significant contribution to HFRS morbidity, with the possible exception of occurrences associated with some type of extreme event, such as war (Plyusnin et al., 1996). In this study, we examined hantaviruses circulating in another location of western Siberia, the Omsk region.

The results of our study show that PUU virus is present in *Arvicolinae* rodents in at least one geographic location of western Siberia. Although this was not surprising, the characteristics of their relationship to other PUU virus strains was unexpected. Our results suggest that hantaviruses from the Omsk region are more closely related to Finnish strains than to Russian strains isolated from geographically closer regions. Phylogenetic analysis of sequence data of

the S-segments clearly shows the monophyly of Finnish and Omsk viruses. Analysis of sequence data of partial M-segments gives similar results. The minor differences that we observed between results of phylogenetic analysis of partial and entire M-segment sequences might be due to sampling error, as just a few sequences of the entire M-segment were available. It was shown before that the phylogenetic analysis of hantavirus M-segment is quite tedious and rarely gives strong branch support values or displays consistent tree topology, especially when closely related taxa are being analyzed (Lundkvist et al., 1998; Asikainen et al., 2000). Another explanation of differences in positioning of the Omsk isolates on the phylogenetic trees might be that selection rate is higher for G1 and G2 glycoproteins than for nucleocapsid protein.

According to a proposed theory (Plyusnin et al., 1995), the extent of hantavirus diversity is proportional to the geographic distance between areas of their circulation. However, while the microgeographical relationships among hantaviruses might be linearly proportional, the macrogeographical relationships are probably more complex. Given the generally accepted theory of co-evolution of hantaviruses and their natural hosts, we might extrapolate a rather complicated natural history of rodents to the evolution of hantaviruses. The macrogeographical evolution of hantaviruses might be significantly biased by distant migrations of the animals, especially ones that occurred during several interglacial epochs.

It is known that there were several cycles of glaciation, with a last warming from the full glacial conditions

18,000 years before present (Hewitt, 2000). During this last warming event, ice, which covered parts of western Europe and Fennoscandia and large areas of Siberia, retreated and species expanded their ranges out of the southern refugia (Hewitt, 1999) northwards. One explanation for our data is that a population of *C. glareolus*, originating in one of these southern refugia, expanded in two directions, one, across the central Russian plain to Finlandia, and another, through the Ural mountain range, to western Siberia. This theory is supported by close similarity of cytochrome b sequences of Omsk and Finnish species of rodents. Previous studies also noted closely related sublineages of hantaviruses from samples obtained in distant geographical locations of Russia (e.g. Moscow region and Bashkortostan) (Ivanov et al., 2000; Nikonorov et al., 2001).

Excessive gene flow from the Omsk region's subpopulation into Finland ($\Theta M = 19.23$; Table 4), observed during multiple runs of "Migrate" program, seems to be implausible due to apparent isolation of the Omsk subpopulation. Perhaps a more valid interpretation of the analyses would be to assign this value not to the Omsk subpopulation but to the originating population located elsewhere, that consisted of haplotypes of rodents which later migrated into target locations in Finland and Omsk region. Because we do not possess any knowledge of genetic composition, size and location of a hypothetical originating population, it was not represented in a data set, therefore forcing the computer program to assign the value to the next most probable source of migration.

There are multiple factors to be considered in population parameters estimates, which might also bias the results, such as presence of indigenous rodents' populations in target locations (although Finland and possibly Omsk locations could be considered "blank", i.e. free of established rodent communities, because those areas were just reemerged from under ice cover). Other factors that are difficult to estimate are the ratio of population sizes of rodents settled in target locations (Finland, Omsk and other possible locations), gene flow from other than the hypothetical originating population, as well as a split point of rodent migration flow and temporal coordinates of a splitting event. We can however speculate that the approximate location of a split point was Nizhniy Novgorod region's vicinity as there is a considerable gene flow ($\Theta M = 3.94$) from this region into the Omsk subpopulation.

Final confirmation of the proposed theory can only be achieved by a study of rodents and hantavirus populations in presumptive southern refugia close to Caucasus and the Black and Caspian seas, which are most likely to be areas where migrating populations, such as those described here, originated. The finding of rodent and viral species that are more closely related to geographically distant counterparts than to nearer ones might be a good argument for this theory.

The close similarity of mitochondrial DNA from bank voles and grey red-backed voles trapped in the Omsk region was first thought to be a result of hybridization between an-

imals, and that this hybridization resulted in "borrowing" of mitochondrial genes. Such introgression of mitochondrial DNA has already been described for species of *Clethrionomys* (Tegelström, 1987; Suzuki et al., 1999), as well as for rodents from different families (Dallas et al., 1995; Fredga and Narain, 2000). However, analysis of nuclear DNA (ncDNA; *Sry-HMG* and *BRCA1* genes), and RAPD analysis, as well as unilateral distribution of cytochrome b sequences (presence of *C. glareolus* haplotype in *C. rufocanus*, and not vice versa) suggests that it is more likely that gray red-backed voles from Omsk region are, in fact, either sympatric species of *C. glareolus* or a subspecies of bank vole. Intraspecific polymorphism, however, seems to be the most probable, as even sympatric species demonstrate certain divergence of mtDNA sequences. We were able to find similar "rutilus" haplotypes of *C. glareolus* both in Bashkortostan and in the Novosibirsk region. Interestingly, at the latter location, the two different haplotypes were found in close proximity—on the opposite banks of the river Ob (Novosibirsk region), one of which, from an eastern bank, was a conventional "glareolus" type (CG12H and CG14H) and another, from a western bank, was a "rutilus" type (CG15H and CG16H). Our data are in good agreement with published results of screening of rodents' mtDNA from the eastern part of European Russia adjacent to the Ural mountains, that includes Bashkortostan, Udmurtia and the Perm region (Morzunov et al., 1999), although no nucleotide sequences from that study have yet been published. Therefore, all these data confirm an existence of hybridization zones in eastern European Russia and western Siberia. Hybridization zones are found across Europe for different species of animals, including bank voles (Jaarola et al., 1999; Fredga and Narain, 2000; Hewitt, 1996), as well as hantaviruses (Hörning et al., 1996).

Interestingly, established and highly active HFRS foci in Eurasia are also located in Fennoscandia, and pre-Ural regions. In those locations, hantaviruses are found both in "rutilus" and in "glareolus" species. Therefore, it is quite possible that such active hantavirus foci appeared as a result of contact between two different rodent haplotypes, one of which might or might not had been carrying a hantavirus. A resulting "bottle-neck" effect, as well as reassortment events, could give a rise to novel, more pathogenic hantaviruses.

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