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Analysis of Puumala hantavirus in a bank vole population in northern Finland: evidence for co-circulation of two genetic lineages and frequent reassortment between strains

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In this study, for the first time, two distinct genetic lineages of Puumala virus (PUUV) were found within a small sampling area and within a single host genetic lineage (Ural mtDNA) at Pallasjärvi, northern Finland. Lung tissue samples of 171 bank voles (*Myodes glareolus*) trapped in September 1998 were screened for the presence of PUUV nucleocapsid antigen and 25 were found to be positive. Partial sequences of the PUUV small (S), medium (M) and large (L) genome segments were recovered from these samples using RT-PCR. Phylogenetic analysis revealed two genetic groups of PUUV sequences that belonged to the Finnish and north Scandinavian lineages. This presented a unique opportunity to study inter-lineage reassortment in PUUV; indeed, 32 % of the studied bank voles appeared to carry reassortant virus genomes. Thus, the frequency of inter-lineage reassortment in PUUV was comparable to that of intra-lineage reassortment observed previously (Razzauti, M., Plyusnina, A., Henttonen, H. & Plyusnin, A. (2008). *J Gen Virol* **89**, 1649–1660). Of six possible reassortant S/M/L combinations, only two were found at Pallasjärvi and, notably, in all reassortants, both S and L segments originated from the same genetic lineage, suggesting a non-random pattern for the reassortment. These findings are discussed in connection to PUUV evolution in Fennoscandia.

INTRODUCTION

Puumala virus (PUUV) (Brummer-Korvenkontio *et al.*, 1980) belongs to the genus *Hantavirus* in the family *Bunyaviridae* (Nichol *et al.*, 2005). Like other members of the genus, PUUV is an enveloped virus with a tri-segmented RNA genome of negative polarity. The small (S) segment encodes the nucleocapsid (N) protein, the medium (M) segment encodes the two surface glycoproteins (Gn and Gc) and the large (L) segment encodes the viral RNA-dependent RNA polymerase (L protein) (Plyusnin *et al.*, 1996; Plyusnin, 2002).

Hantaviruses are zoonotic pathogens that cause haemorrhagic fever with renal syndrome (HFRS) throughout Eurasia and hantavirus (cardio)pulmonary syndrome in the Americas (Schmaljohn & Hjelle, 1997). More than 5000 cases of nephropathia epidemica, a relatively mild form of HFRS caused by PUUV, are diagnosed annually in Europe (Heyman & Vaheri, 2008).

The GenBank/EMBL/DDBJ accession numbers for the sequences of PUUV S, M and L are FJ717661–FJ717679.

The natural host of PUUV is the bank vole, *Myodes glareolus*, which belongs to the subfamily *Arvicolinae* of the family *Cricetidae* (Wilson & Reeder, 2005). The bank vole is widely distributed throughout Europe, from the British Isles to the Urals, excluding some northernmost regions and the Mediterranean coast. Its range continues eastward into central Siberia (IUCN, 2007). Throughout its distribution, the genetic variants of PUUV show phylogeographical clustering (Plyusnin *et al.*, 1994, 1995a; Hörling *et al.*, 1996; Lundkvist *et al.*, 1998; Asikainen *et al.*, 2000; Sironen *et al.*, 2001; Johansson *et al.*, 2008). Bank voles recolonized Fennoscandia after the last glaciation period, 12 000–9000 years ago, from different directions (Fig. 1). The Western European bank vole lineage, defined by mitochondrial DNA (mtDNA), was confined in a Central European glacial refugium (Deffontaine *et al.*, 2005). This lineage migrated to Fennoscandia through a land bridge, at that time connecting Denmark and Sweden, and colonized southern Norway and Sweden (Björck, 1995, 1996; Jaarola & Tegelström, 1996). The Eastern European bank vole lineage expanded from the environs of the Carpathian Mountains towards northern Europe up to central Finland

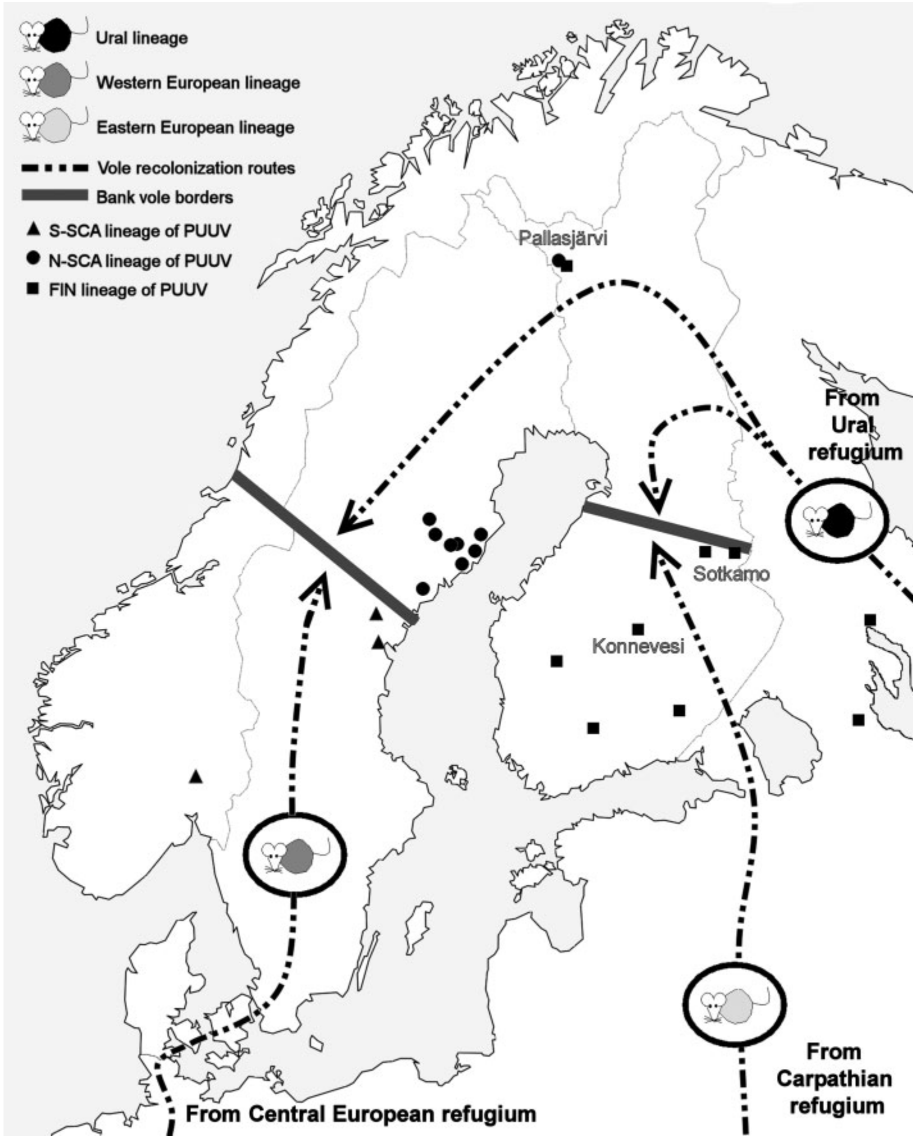


Fig. 1. Recolonization routes of bank voles to Fennoscandia and the distribution of PUUV genetic lineages.

(Deffontaine *et al.*, 2005; Kotlík *et al.*, 2006; Deffontaine-Deurbroeck, 2008). The Ural bank vole lineage, which carries the mtDNA of the red vole (*Myodes rutilus*) (Tegelström, 1987), moved from the southern Urals over northern Russia towards north Fennoscandia (Deffontaine-Deurbroeck, 2008). The Ural lineage, migrating from the north-east, met the Western European lineage in north-central Sweden/Norway and the Eastern European lineage in central Finland, establishing two contact zones (Jaarola *et al.*, 1999) (Fig. 1).

It seems that each of the above-mentioned bank vole lineages carried its own distinct genetic lineage of PUUV. As a result, three PUUV lineages are currently found in Fennoscandia: (i) the south Scandinavian (S-SCA) lineage represented by strains from south Norway and central Sweden up to the contact zone in north-central Sweden; (ii) the Finnish (FIN) lineage comprising strains from south-central Finland and Russian Karelia; and (iii) the north Scandinavian (N-SCA) lineage from northern Sweden (Fig. 1). Other known PUUV lineages are: the central European lineage, which includes strains from Germany, Belgium, France and Slovakia; the Alpe-Adrian lineage, which comprises strains from Austria, Slovenia and Croatia; the Danish lineage in Denmark; and the Russian lineage with strains from central Russia (Plyusnina *et al.*, 2006; Razzauti *et al.*, 2008 and references therein).

The genetic diversity of PUUV is generated mostly by genetic drift: accumulation of nucleotide substitutions throughout the genome as well as small deletions and insertions within the non-coding regions of the RNA segments caused by errors of the viral RNA polymerase (Plyusnin *et al.*, 1996; Plyusnin, 2002). There is also some evidence for genome reassortment (Plyusnin *et al.*, 1997; Razzauti *et al.*, 2008) and recombination (Sironen *et al.*, 2001) in PUUV. The steady generation of mutant sequences during PUUV replication in an individual host results in a swarm of viral quasi-species (Plyusnin *et al.*, 1995b), an ensemble of closely related sequences that cooperates in maintaining distinct features of a virus such as its transmissibility and pathogenicity (Domingo & Holland, 1997; Vignuzzi *et al.*, 2006). In general, PUUV evolution follows the neutral (or quasi-neutral) mode and the estimated evolution rate appears to be relatively low (Sironen *et al.*, 2001).

In a previous study of PUUV in a bank vole population at Konnevesi, central Finland, we showed that accumulation of point mutations and reassortment of the genomic RNA segments were involved in PUUV microevolution (Razzauti *et al.*, 2008). It should be emphasized that the genetic diversity of the Konnevesi PUUV strains was relatively high: up to 4.9 and 4.8 % for the S and M segments, respectively, and even 9.7 % for the L segment. This allowed a simple definition of genetic variants and easy detection of reassortant genomes. In the present study, we selected a bank vole population at Pallasjärvi in the Pallas-Ylläs National Park in northern Finland. Bank voles from this region belong to the Ural mtDNA lineage, in contrast with the Konnevesi bank vole population, which belongs to the Eastern European mtDNA lineage (Deffontaine-Deurbroeck, 2008). Bank voles in these two regions also differ in seasonal and multi-annual density dynamics (Hansson & Henttonen, 1985, 1988; Henttonen, 2000), which could influence the overall pattern of PUUV transmission among rodents. Our earlier observations confirmed circulation of PUUV at Pallasjärvi (T. Sironen, H. Henttonen & A. Plyusnin, unpublished data). The aims of the current study were to learn about PUUV micro-evolution at the contact zone of two PUUV lineages within a single host lineage and to compare the genetic diversity of the virus and the frequency of the genome segment reassortment at Konnevesi and Pallasjärvi.

METHODS

Sampling of rodents and screening of rodent samples. Rodents were trapped at Pallasjärvi in the Pallas-Ylläs National Park in western Finnish Lapland (68° 30′ N 24° 09′ E) in September 1998. Bank vole densities in that year were typical (for long-term rodent studies at Pallasjärvi, see, for example, Henttonen *et al.*, 1987; Henttonen, 2000). For this study, bank voles were trapped alive and euthanized the same day, and tissue samples were stored in liquid nitrogen. The samples were first screened for PUUV N antigen by immunoblotting as described previously (Plyusnin *et al.*, 1995a).

RT-PCR and sequencing. Viral RNA was extracted from lung tissue samples of the N antigen-positive bank voles using TriPure reagent (Boehringer Mannheim) according to the manufacturer's instructions. Reverse transcription was performed with SuperScript II reverse transcriptase (Invitrogen/Gibco-BRL) as specified by the manufacturer. For PCR, AmpliTaq DNA polymerase (Perkin-Elmer/Roche Molecular Systems) was used. RT-nested-PCR was performed essentially as described previously (Razzauti *et al.*, 2008), using described primers unless specified below. Both reverse transcription and the first PCR were carried out with primers Sa31 and PUU5 for the S segment, with the primers MFPuu1793 (5′-AATCCATCTGA-GGCWACAMCRTC-3′) and MRPuu3011 (5′-CCRACWCCTGAA-CCCCATGC-3′) for the M segment and PUULF1 and PUULR2 for the L segment. For the nested PCRs, the primers PUUS7 and Sa5 were used for the S segment, primers MFPuu2139 (5′-AGTTACAGAAT-CCTGCWAATGA-3′) and MRPuu2651 (5′-TGRCATGTTGTWG-TRCACCATTG-3′) for the M segment, and primers PUULF2 and PUULR2 for the L segment. The PCR amplicons of 502 bp for the S segment, 486 bp for the M segment and 522 bp for the L segment were separated by electrophoresis in a low-melting-point agarose gel (peqGOLD Low Melt Agarose; PEQLAB) and purified with a QIAquick gel extraction kit (Qiagen). Sequencing was performed automatically by using an ABI PRISM dye terminator sequencing kit (Perkin Elmer/ABI).

Analysis of bank vole mtDNA. DNA was purified from lung tissue samples using TriPure reagent (Boehringer Mannheim) as specified by the manufacturer. The D-loop region of mtDNA was amplified as described by Morzunov *et al.* (1998) using primers CBT-MR1 and MD1-12STC.

Phylogenetic analyses. Sequence handling was carried out with BioEdit version 7.0.9 (Hall, 1999) and multiple sequence alignments were prepared by using CLUSTAL W version 1.4 (Thompson *et al.*, 1994) with default parameters. For comparison, PUUV genome sequences and the sequences of other hantaviruses were retrieved from GenBank. The PHYLIP program package (Felsenstein, 1993) was used to make 1000 bootstrap replicates of the sequence data (SEQBOOT program). Distance matrices were calculated by using the F84 model for nucleotide substitution (DNADIST program) and analysed by using Fitch–Margoliash (FM) tree-fitting algorithms. The bootstrap support values for particular branching points were calculated from these trees using the CONSENSE program. The resulting trees were viewed with TreeView (version 3.4.0).

RESULTS

Screening of rodent samples and analysis of bank vole mtDNA

Lung tissue samples of 171 bank voles were screened for the presence of PUUV N antigen by immunoblotting and 25 were found to be positive. The presence of PUUV genome RNA was later confirmed in all of these samples by RT-PCR. For mtDNA analysis, the D-loop region was amplified and sequenced from six bank vole tissue samples. All six sequences matched the mtDNA of the red vole (*M. rutilus*), confirming previous observations that the bank voles from Pallasjärvi belong to the Ural bank vole lineage.

Genetic analysis of wild-type PUUV strains

Partial sequences of the PUUV S, M and L genome segments were recovered from all 25 N antigen-positive samples. The characteristics of the mutations found are summarized in Table 1. The majority of mutations were located at the third position of codons suggesting strong

Table 1. Characteristics of mutations observed in Pallasjärvi PUUV strains

Genome segments	S (nt 640–1082)	M (nt 2180–2632)	L (nt 577–1032)
Total number of mutations	86	85	93
Transitions (ts)/transversions (tv)	49/37	56/29	52/41
Most frequently occurring ts	C→U (36.7 %)	A→G (35.7 %)	A→G (32.7 %)
Most frequently occurring tv	U↔A (45.9 %)	U↔A (41.4 %)	U↔A 46.3 %)
Distribution of mutations in codon positions 1/2/3	11/1/74	7/4/74	15/4/74

stabilizing (negative) selection working against changes in the viral proteins. All S segment sequences could be assigned to five genetic variants that formed two distinct groups: S1/S2 and S3–S5 (Fig. 2). Within the groups, the genetic diversity of PUUV variants was modest, but diversity between the groups appeared to be surprisingly high (Table 2). Analysis of the deduced N protein sequences gave similar results. No amino acid substitutions were seen within the second group and only one (Val260Ile) was seen within the first group. In contrast, when the N sequences from the two groups were compared, 11 aa substitutions were observed, seven of which were conservative. Three of these 11 residues belonged to lineage-specific amino acid signatures (Sironen *et al.*, 2001); they were found in genetic variants from the FIN (Met262, Asp304) and the N-SCA (Asp272) lineages. These findings suggested that the two genetic groups of Pallasjärvi PUUV sequences belonged to different genetic lineages: the FIN (genetic variants S1/S2) and the N-SCA (variants S3–S5) lineages. Our phylogenetic analysis (Fig. 3a) showed that this was indeed the case.

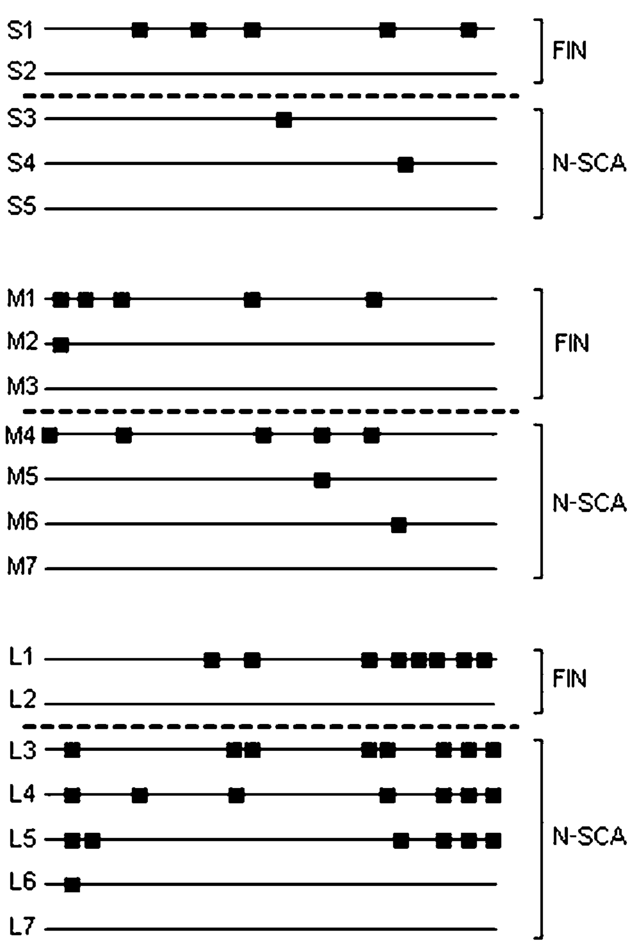


Fig. 2. Nucleotide mutations in Pallasjärvi PUUV variants. Mutations (filled squares) in each of the two lineages are shown versus the prototype sequences of Pallas80 (S2/M3/L2) for the FIN lineage and Pallas90 (S5/M7/L7) for the N-SCA lineage. For the clarity of presentation, mutations between the lineages are not shown: 81 mutations in the S segment, 76 mutations in the M segment and 77 mutations in the L segment.

All M sequences could be arranged into seven genetic variants (Table 2, Fig. 2). Similar to the S sequences, they formed two distinct groups with relatively low intragroup but high intergroup diversity. Partial Gc sequences were identical for all variants within the lineages, but differed at eight positions between lineages; 6 of these 8 aa substitutions were conservative. Phylogenetic analysis showed that genetic variants M1–M3 from the first group belonged to the FIN genetic lineage, whilst the variants M4–M7 from the second group belonged to the N-SCA lineage (Fig. 3b). The L segment sequences constituted seven genetic variants (Table 2, Fig. 2, which again formed two groups (L1/L2 and L3–L7) with low intragroup but high intergroup diversity. Similar to the S and M segments, the two groups of L segment sequences belonged to the FIN and N-SCA lineages (Fig. 3c). No amino acid substitutions were seen within the deduced L protein sequences of the N-SCA lineage and only one (Val260Ile) was seen within the sequences of the FIN lineage. Between the lineages, 11 aa substitutions were found, seven of which were conservative.

Taken together, these results showed co-circulation of PUUV strains belonging to two genetic lineages in the study area at Pallasjärvi. This presented a unique opportunity to study the inter-lineage reassortment in PUUV.

PUUV genome segment reassortment

PUUV strains found in 25 positive bank voles were designated Pallas/Mg2/1998 to Pallas/Mg141/1998 (herein

Table 2. Genetic diversity of PUUV variants at Pallasjärvi

Genome segment*	S	M	L
No. of genetic variants	5	7	7
No. of genetic groups (no. of sequences, group 1/group 2)	2 (12/13)	2 (18/7)	2 (12/13)
Frequency of nt substitutions (*n*) (%)			
Group 1	5 (1.1)	5 (1.1)	8 (1.8)
Group 2	2 (0.5)	6 (1.3)	11 (2.4)
Group 1 vs group 2	85 (19.2)	83 (18.3)	88 (19.3)
Genetic diversity (%)			
Group 1	1.1	0.9–1.1	1.7
Group 2	0.2	0.2–1.1	0.2–1.7
Group 1 vs group 2	18.3–19.4	17.1–19	16.7–20.2
Frequency of deduced aa (*n*) (%)			
Group 1	1 (0.7)	0	0
Group 2	0	0	1 (0.7)
Group 1 vs group 2	11 (7.5)	8 (5.3)	11 (7.2)

*Group 1 belongs to the FIN lineage and group 2 to the N-SCA lineage.

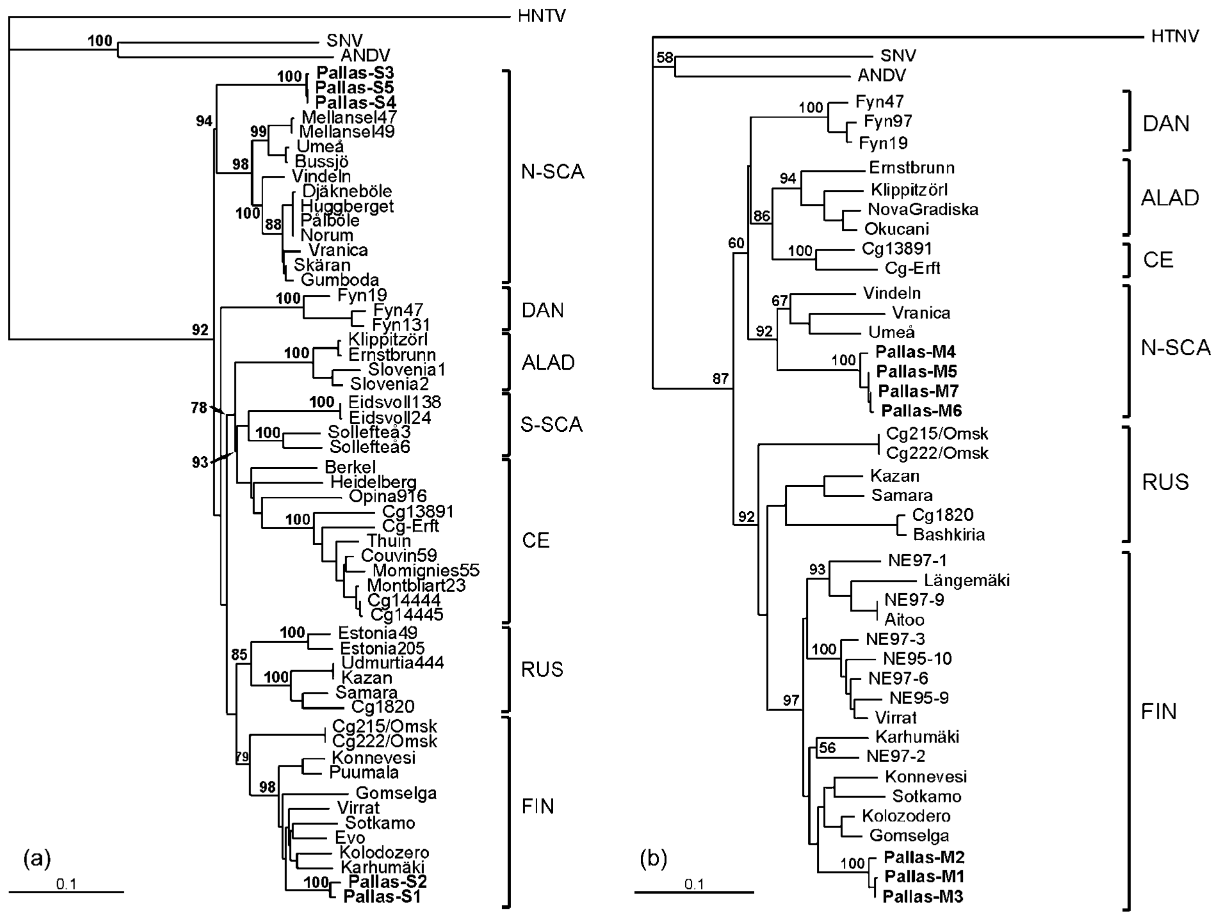
referred to as Pallas2 to Pallas141). As some of the recovered partial sequences were identical, the number of distinct novel PUUV genomes totalled 13 (Table 3). Analysis of the phylogenetic clustering and distribution of point mutations in the recovered PUUV sequences revealed that genetic variants belonging to different groups/lineages might reassort their genome segments. For example, the S and L segments of the Pallas134 strain belonged, respectively, to the S4 and L4 variants of the N-SCA lineage, whilst the M segment belonged to the M2 variant of the FIN lineage (Table 3). Six of the 13 newly characterized strains (46.2 %) possessed reassortant genomes and, taking into account identical sequences, eight of the 25 infected bank voles (32 %) carried reassortant viruses. Of the six possible reassortant S/M/L combinations, only two were found and, notably, in all reassortants both S and L segments originated from the same genetic lineage (Table 3). One strain possessed PUUV genomes of the $S_{FIN}M_{N\text{-}SCA}L_{FIN}$ type and five of the $S_{N\text{-}SCA}M_{FIN}L_{N\text{-}SCA}$ type. It is worth mentioning that a particular variant of the M segment, M2, was found in the majority of bank voles infected with strains from FIN lineage but in only one of five reassortants (Table 3). Taken together, these observations suggest a non-random pattern for reassortment.

DISCUSSION

Co-circulation and interaction of two genetic lineages of PUUV

In this study, two distinct PUUV lineages, FIN and N-SCA, were found within a small sampling area and within a single host genetic lineage. The contact zone of the N-SCA and FIN PUUV lineages is so far unknown. When studying strains of PUUV in bank voles at Pallasjärvi, we observed two genetic groups for each of the three viral RNA genome segments. What at first glance looked like a rather variable set of sequences turned, after further genetic and phylogenetic analyses, into two clusters belonging to distinct PUUV lineages. This is the first time that two PUUV lineages have been found co-circulating within the same host population. It is well established that the contact zone for the Ural and Western European bank vole lineages in north-central Sweden is congruent with the contact zone of the S-SCA and the N-SCA PUUV lineages (Hörling *et al.*, 1996; Johansson *et al.*, 2008; K. Nemirov & Å. Lundkvist, personal communication) (Fig. 1). In Finland, the contact zone between the Ural and Eastern European bank vole lineages is several hundred kilometres south of our study area at Pallasjärvi (Deffontaine-Deurbroeck, 2008). So far, the northernmost published Finnish PUUV sequence was from Sotkamo (64° 7′ N 28° 22′ E; Fig. 1). Our observations raise an interesting question: how (and when) were PUUV genetic variants of the FIN lineage transferred to the Ural bank vole lineage? One possibility could be that the virus gradually spread northwards via rodent-to-rodent contact, for example during mating seasons when males can travel relatively long distances (up to 2–3 km) looking for female partners.

It seems that two PUUV lineages have been co-circulating at Pallasjärvi for some time and yet neither has outcompeted the other. There are three possible reasons for this: (i) the lineages have approximately the same fitness; (ii) competition between the lineages is not strong enough to oust the other; (iii) highly competitive (or even dominant) variants of the FIN lineage arrived only recently in the area. The above-mentioned possibility that these variants have spread to the north from the host contact zone would support this third alternative. Based on a relatively small number of infected bank voles from the



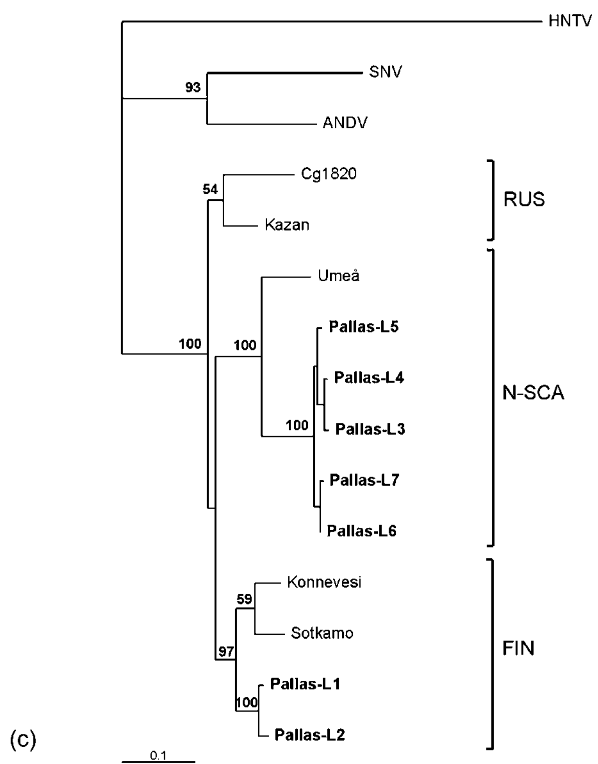


Fig. 3. Phylogenetic (FM) trees calculated for the partial S, M and L segment sequences of PUUV. (a) S segment sequences (nt 640–1082), (b) M segment sequences (nt 2180–2632) and (c) L segment sequences (nt 577–1032). Bootstrap support values were calculated for 1000 replicates; only values greater than 50 % are shown. Hantaan (HTNV), Andes (ANDV) and Sin Nombre (SNV) viruses were used as representatives of other hantaviruses. Lineages: DAN, Danish; ALAD, Alpe-Adrian; CE, Central European; RUS, Russian. Bars, 0.1 substitutions per site.

Table 3. Types of individual PUUV genome combinations

No. of sequences observed	Sample no.*	S	M	L	Lineage
11	68, 76, 82, 106, 108, 112, 113, 117, 127, 130	S1	M2	L1	FIN (two variants)
	80	S2	M3	L2	
6	2	S3	M4	L3	N-SCA (five variants)
	9	S5	M5	L6	
	90	S5	M7	L7	
	91, 96	S4	M6	L7	
	141	S4	M4	L6	
7	4, 11	S3	M3	L3	$S_{N\text{-}SCA}/M_{FIN}/L_{N\text{-}SCA}$ (five reassortant variants)
	66, 137	S5	M3	L6	
	97	S4	M3	L7	
	109	S4	M1	L5	
	134	S4	M2	L4	
1	63	S1	M6	L1	$S_{FIN}/M_{N\text{-}SCA}/L_{FIN}$ (one reassortant variant)

*Sample nos correspond to PUUV strains from Pallas2 to Pallas141.

collection of 1998, it is impossible to say whether either of the two lineages showed more success in infecting bank voles. Strains of the FIN lineage were found in 11 animals, whilst strains of the N-SCA lineage were found in six; the difference does not look substantial. To clarify these points, we initiated a multi-annual analysis of the dynamics of PUUV lineages in bank voles collected in the same study area at Pallasjärvi annually from 1998 to the present.

As expected, the inter-lineage genetic diversity of Pallasjärvi PUUV strains was high: 18.3–20.2 %. The intra-lineage diversity, however, appeared to be considerably lower (0.2–1.7 %) in comparison with what we observed previously in central Finland (Razzauti *et al.*, 2008) or what was reported in northern Sweden (Johansson *et al.*, 2008). Could this be due to the presence of two lineages sharing the same host population? It would be logical to assume that the joint pool of genetic variants from two co-circulating lineages should be twice as large as the pool of genetic variants for each of them. Obviously, this is not the case. One can speculate that the presence of two lineages exerts a somewhat stronger selection pressure, and thus only the best-fit variants of each lineage remain in circulation.

Inter-lineage genome segment reassortment in PUUV

Co-circulation of both FIN and N-SCA PUUV strains within a relatively small study area presented an opportunity to trace possible exchanges of PUUV genome material between these two lineages. Indeed, we observed a substantial proportion of reassortants among the sequenced virus genomes (8/25; 32 %). Thus, the frequency of inter-lineage PUUV reassortment was comparable to the 39 % found for Sin Nombre virus (SNV), although the study area for SNV was much larger (Henderson *et al.*, 1995), and also to the frequency of PUUV intra-lineage reassortment observed in our previous study (20 %) in central Finland (Razzauti *et al.*, 2008).

In the studied bank vole population at Pallasjärvi, only two of six possible reassortant types were found and, in both of these, the S and L segments originated from the same genetic lineage (Table 3). This pattern was seen previously with SNV reassortants (Henderson *et al.*, 1995). Our preliminary data on the PUUV strains collected at Konnevesi in 2008 indicate a similar trend. However, our data on the Konnevesi PUUV collection of 2005 showed more frequently homologous association of the M and L segments (Razzauti *et al.*, 2008). Of course, in all of these studies, the number of analysed reassortants was not high. It therefore remains to be seen whether there is a stable pattern of reassortment in hantaviruses (i.e. non-random exchange of RNA genome segments between different lineages or genetic groups within the same lineage), which could reflect unequal fitness, and hence different survival rates, of newly generated gene combinations. So far, the survival of individual reassortant PUUV genomes or distinct type(s) of reassortants has not been followed locally from one year to another. Consequently, it is not known whether certain reassortants could survive for several seasons/years in the virus genetic pool. Nevertheless, it seems safe to assume that constant generation of reassortants does not present a threat to the existence of parental PUUV lineages: apparently, they are not overrun by the reassortant offspring and sustain their presence in a host population.

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