

Molecular evolution of Puumala hantavirus in Fennoscandia: phylogenetic analysis of strains from two recolonization routes, Karelia and Denmark

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Like other members of the genus *Hantavirus* in the family *Bunyaviridae*, Puumala virus (PUUV) is thought to be co-evolving with its natural host, the bank vole *Clethrionomys glareolus*. To gain insight into the evolutionary history of PUUV in northern Europe during the last post-glacial period, we have studied wild-type PUUV strains originating from areas along two postulated immigration routes of bank voles to Fennoscandia. Full-length sequences of the S RNA segment and partial sequences (nt 2168–2569) of the M segment were recovered by RT-PCR directly from bank vole tissues collected at three locations in Russian Karelia and one location in Denmark. Phylogenetic analysis showed that strains from Karelia and Finland belong to the same genetic lineage, supporting the hypothesis that PUUV spread to present Finland via a Karelian land-bridge. The Danish PUUV strains showed no particularly close relatedness to any of the known PUUV strains and formed a distinct phylogenetic lineage on trees calculated for both S and M segment sequences. Although no direct link between the Danish PUUV strains and those of the southern Scandinavian lineage was found, within the S segment of Danish PUUV strains, two regions with higher similarity to either northern Scandinavian or – to a less extent – southern Scandinavian genetic lineages were revealed, suggesting evolutionary connections of their precursors.

Introduction

Puumala virus (PUUV) is a member of the genus *Hantavirus* in the family *Bunyaviridae*. The genome of PUUV consists of three single-stranded RNA segments of negative polarity: large (L) of 6.5 kb, medium (M) of 3.7 kb, and small (S) of

1.8 kb in size, encoding a viral RNA polymerase, two surface glycoproteins G1 and G2, and a nucleocapsid protein (N), respectively (Elliott *et al.*, 1991). PUUV is the causative agent of a mild form of haemorrhagic fever with renal syndrome, nephropathia epidemica, which affects humans in northern and central Europe and western parts of Russia (for review, see Plyusnin *et al.*, 1996). The virus is associated with bank voles (*Clethrionomys glareolus*), which belong to the subfamily *Arvicolinae* of the family *Muridae*. In the host rodents, the virus causes a life-long persistent asymptomatic infection.

The bank vole is found in central Europe and most parts of Fennoscandia (this collective term is used for Finland and Scandinavia) and western Russia, excluding the northern-most parts of Lapland (Mitchell-Jones *et al.*, 1999). Two types of bank voles, northern and southern, can be distinguished in Fennoscandia; the mtDNA of the northern population origi-

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The nucleotide sequence data (consensus sequences) reported in this paper have been deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession nos AJ238788–AJ238791, AJ278086–AJ278087 and AJ278089–AJ278093.

nated from a different species, the red vole (*Clethrionomys rutilus*). It is assumed that the mtDNA transfer took place 8000–13 000 years ago during the post-glacial recolonization of Fennoscandia by a plethora of plant and animal species (Tegelström, 1987). While retreating from Fennoscandia, the Late Weichselian continental glacier left two potential immigration routes for animals and plants to recolonize the uncovered land. The southern route was via present Denmark and southern Sweden (when there was a land connection between them), while the eastern route was via present Russia and Finland. The northern and the southern populations of bank voles met in central Sweden, forming a contact zone, which is still approximately 50 km wide; similar contact zones for the field vole (*Microtus agrestis*) and common shrew (*Sorex araneus*) were found in the same area (for a review, see Jaarola *et al.*, 1999). Notably, PUUV strains carried by bank voles north and south of the contact zone form two distinct phylogenetic lineages (Hörling *et al.*, 1996; Lundkvist *et al.*, 1998), thus supporting the hypothesis of a hantavirus–host co-evolution (Plyusnin *et al.*, 1996; Morzunov *et al.*, 1998; Vapalahti *et al.*, 1999).

In this study, we have analysed wild-type (wt) PUUV strains originating from Russian Karelia and Denmark, locations along the two postulated recolonization routes to Fennoscandia after the last ice age. Our aim was to learn about the relationships of these strains and known phylogenetic lineages of PUUV from Europe.

Methods

■ Rodents. Animal experimentation guidelines approved by the American Society of Mammalogists, Animal Care and Use Committee (1998) were followed in animal studies. In Karelia (Russia Federation), bank voles were trapped in August 1995 with live-traps at three localities around Lake Onega: Karhumäki (Medvedegorsk), Gomselga and Kolodozero (north, west and east of Lake Onega, respectively) (Fig. 1C). Voles were sacrificed and dissected for lung and/or liver tissue. Aliquots (approximately 50 µl) of lung tissue samples were put into either 500 µl of Laemmli sample buffer (Laemmli, 1970) or 1 ml of D-solution (Chomczynski & Sacchi, 1987) for immunoblotting and RNA extraction, respectively. The samples were transported in liquid nitrogen to Haartman Institute and stored at –70 °C until analysed for the presence of PUUV N antigen (Ag) as described previously (Plyusnin *et al.*, 1995). Briefly, samples were thawed on ice and homogenized by sonication in Laemmli sample buffer for 1 min at room temperature. Aliquots of 10 µl were separated by electrophoresis in 10% SDS–polyacrylamide gels and immunoblotted with polyclonal antibody (Ab) raised against recombinant PUUV N protein (Vapalahti *et al.*, 1995).

In Denmark, voles were live-trapped in three locations, Jutland and the islands of Sealand and Fyn, in April 1990. The animals were bled, and liver, kidney and lungs were dissected. The Ab-positive samples were selected by an indirect immunofluorescence test using PUUV-infected Vero cells (Niklasson & LeDuc, 1987). Tissue samples were stored at –80 °C until analysed.

■ RT–PCR, cloning and sequencing. RNA isolation, RT–PCR and cloning were done as described previously (Plyusnin *et al.*, 1994). Briefly, total RNA was isolated from the tissue samples by the acid guanidinium

thiocyanate–phenol–chloroform method of Chomczynski & Sacchi (1987). Full-length S segment cDNAs were synthesized with Superscript (Bethesda Research Laboratories) or AMV (Boehringer Mannheim) reverse transcriptase in the presence of primer 5' TAGTAGTA-(G/T)(A/G)C 3' and random hexamers. PCR was done with a single primer 5' TTCTGCAGTAGTAGTAGACTCCTTGAAAAG 3' and the PCR products corresponding to the full-length S segment (~1830 nt) were cloned into the pGEM-T plasmid vector with a TA cloning kit (Promega) using the procedure recommended by the manufacturer. Plasmids were purified with a Wizard Mini-preps kit (Promega) or a QIAprep kit (QIAGEN) and sequenced with either Sequenase version 2.0 (Amersham Life Science) or automatically. In the latter case, sequencing was performed using either ABI PRISM Dye Terminator or ABI PRISM M13F and M13R Dye Primer sequencing kits (Perkin Elmer).

RT of the partial M segment was performed as described previously (Plyusnin *et al.*, 1997) with MMLV reverse transcriptase (Amersham) in the presence of primers A1 (5' AATCCATCTGAGGCTACACCGTCT 3', nt 1793–1816) and C2 (5' CCAACTCCTGAACCCCATGC 3', nt 3011–3030). PCR was done with the same primers, A1 and C2, and nested PCR was done using primers B1 (5' AACCCGGCAAT-GAACAAGAA 3', nt 2147–2167) and B2 (5' TTGGTTGGAGAG-GACCGAGGAAT 3', nt 2611–2632) for the Karelian samples and B3 [5' CA(A/G)TTACA(A/G)AA(T/C)CCIGC(C/A)AATGA 3', nt 2138–2160] and B4 (5' TGAATTTTGAACAGTTCCTCA 3', nt 2571–2592) for the Danish samples. Amplified products were gel-purified using QIAquick Gel Extraction kit (QIAGEN) and sequenced automatically.

■ Multiple sequence alignments. Alignments were prepared with ClustalX (Thompson *et al.*, 1997). The following options were used: gap opening, 15; gap extension, 6.66; delay divergent sequences, 40%; DNA transition weight, 0.50; no negative matrix. Minor corrections to the alignment were introduced manually using the SeqApp 1.9a169 sequence editing program (Gilbert, 1992). Alignment of the partial M segment was done manually using SeqApp.

■ Phylogenetic analyses. The GCG software package was used for sequence entry and analysis (version 10.1). The PHYLIP program package (Felsenstein, 1993) was used to analyse the sequence data: 500 bootstrap replicates (Seqboot program) were fed to the distance matrix algorithm (Dnadist program, with Kimura's two-parameter option); distance matrices were analysed with either the Fitch–Margoliash or the Neighbour-joining tree fitting algorithms; the bootstrap support for the trees were calculated with the Consense program. The nucleotide sequence data were also analysed with maximum likelihood (DNAML) and maximum parsimony (DNAPARS) algorithms from the PHYLIP program (Felsenstein, 1993).

The trees for deduced protein sequences were calculated by first translating the nucleotide sequences into amino acid (aa) sequences with SeqApp 1.9a169 (Gilbert, 1992). Then the PHYLIP package (Felsenstein, 1993) was used to make 500 bootstrap replicates which were fed to the distance matrix (Protdist) and maximum parsimony (Protpars) algorithms. Distance matrix data were analysed with a Neighbour-joining tree fitting algorithm. The bootstrap support was calculated with the Consense program.

For comparison, the following hantavirus sequences were used. (i) S segment/N protein: Puumala virus, strain Sotkamo (GenBank accession no. X61035), Evo/12Cg/93 (Z30702), Evo/13Cg/93 (Z30703), Evo/14Cg/93 (Z30704), Evo/15Cg/93 (Z30705), Virrat/25Cg/95 (Z69985), Puumala/1324Cg/79 (Z46942), Eidsvoll/1124v (AJ223368), Eidsvoll/Cg1138/87 (AJ223369), Sollefteå/Cg3/95 (AJ223376), Sollefteå/Cg6/95 (AJ223377), Hundberget/Cg36/95 (AJ223371), Mellansel/Cg47/94 (AJ223374), Mellansel/Cg49/95 (AJ223375), Tavelnsjö/Cg81/94 (AJ-

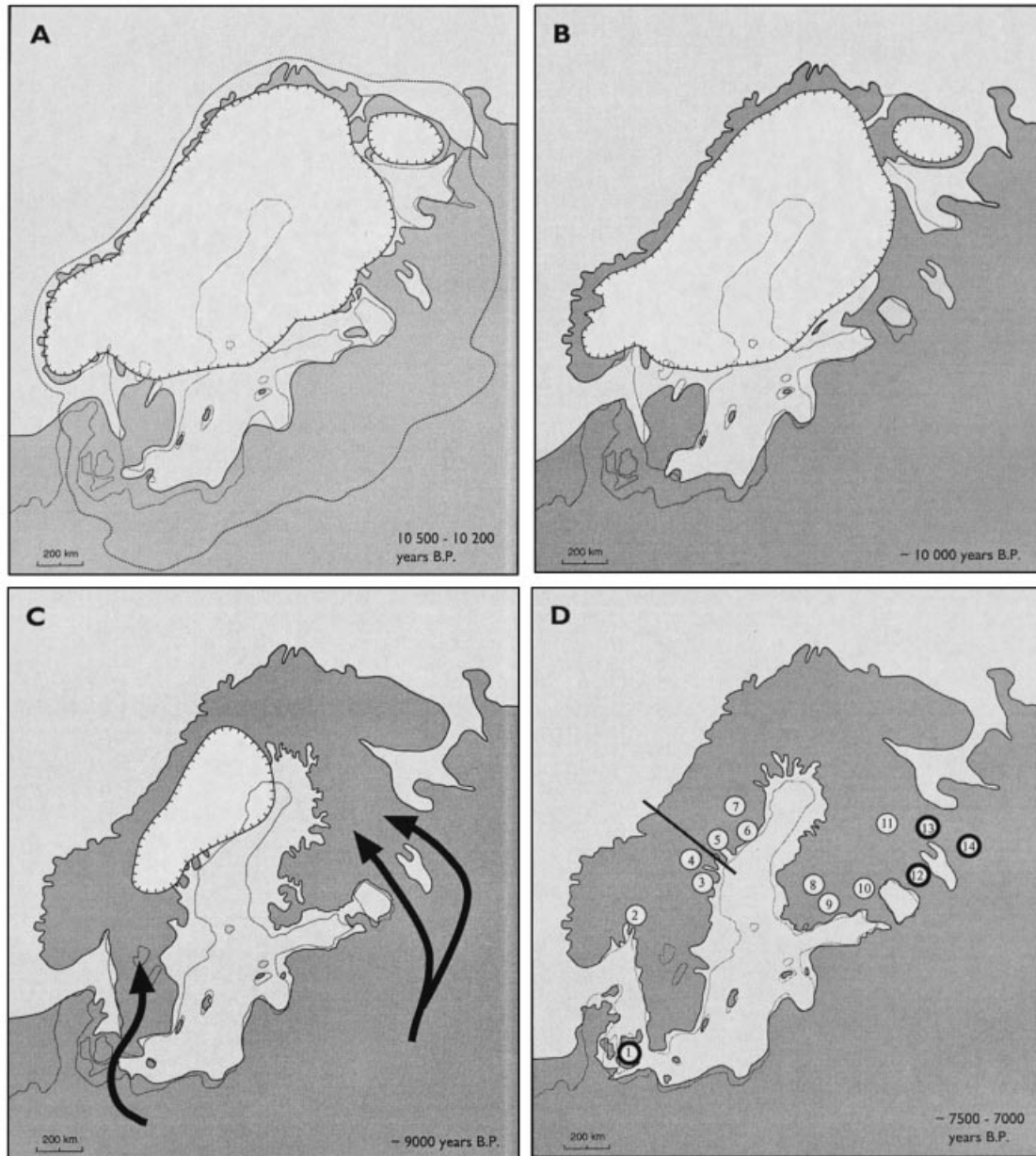


Fig. 1. Late Weichselian deglaciation of the Fennoscandian area and the trapping locations. (A) 10 500–10 200 years before present (B. P.). (B) ~ 10 000 years B. P. (C) ~ 9 000 years B.P. Arrows show postulated migration routes. (D) ~ 7 500–7 000 years B.P. 1, Fyn; 2, Eidsvoll; 3, Sundsvall; 4, Sollefteå; 5, Mellansel; 6, Tavelsjö/Vindeln; 7, Hundberget; 8, Virrat; 9, Evo; 10, Puumala; 11, Sotkamo; 12, Gomselga; 13, Karhumäki; 14, Kolodozero.

223380), Vindeln/L20Cg/83 (Z48586), Vindeln/Cg4/94 (AJ223381), 'Vranica'/Hällnäs (U14137), Udmurtia/338Cg/92 (Z30708), Udmurtia/444Cg/88 (Z30706), Udmurtia/458Cg/88 (Z30707), Udmurtia/894Cg/91 (Z21497), Kazan (Z84204), Cg1820 (M32750), P360 (L11347), Cg13891 (U22423), Tobetsu-60Cr-93 (AB010731) and Kamiiso-8Cr-95 (AB010730). Other hantaviruses: Tula virus strain Moravia02v (Z69991); Hantaan virus, strain 76-118 (M14626); Dobrava virus, strain Dobrava (L41916); Dobrava virus, strain Saaremaa/160V (AJ009773); Seoul virus, strain SR-11 (M34881); El Moro Canyon virus, strain RM-97 (U11427); New York virus, strain RI-1 (U11427); Sin Nombre virus, strain NM H10 (L25784); Bayou virus, strain Louisiana

(L36929); Black Creek Canal virus (L39949); Laguna Negra virus, strain 510B (AF005727); Topografov virus, strain Ls136V (AJ011646); Khabarovsk virus, strain MF-43 (U35255); and Prospect Hill virus, strain PH-1 (Z49098). (ii) M segment/G2 protein: Puumala virus, strain Sotkamo (GenBank accession no. X61034), Virrat (Z70201), NE9/95 (Z69988), NE97-1 (AJ238141), NE97-2 (AJ238142), NE97-3 (AJ238143), NE97-6 (AJ238144), NE97-9 (AJ238145), NE10/95 (Z69990), Längemäki/126Cg/97 (AJ238139), Aitoo/84Cg/97 (AJ238140), 'Vranica'/Hällnäs (U14136), VindelnL20 (Z49214), Kazan (Z84205), Bashkiria-Cg1820 (M29979), Cg 13891 (U22418), Cg-Erft (AJ238778). Other hantaviruses: Bayou virus, strain Louisiana (L36930); Black Creek Canal

virus (L39950); El Moro Canyon virus, strain RM-97 (U26828); Laguna Negra virus, strain 510B (AF005728); Sin Nombre virus, strain NM H10 (L25783); New York virus, strain RI-1 (U36801); Blue River virus, strain Indiana (AF030551); Seoul virus, strain SR-11 (M34881); Dobrava virus, strain Saaremaa/160V (AJ009774); Dobrava virus, strain Dobrava (L33685); Hantaan virus, strain 76-118 (M14267); Thailand virus, strain 749 (L08756); Prospect Hill virus, strain PH-1 (Z55129); Tula virus, strain Moravia/5302v/95 (Z69993); Topografov virus, strain Topografov/Ls136V (AJ011646); and Khabarovsk virus, strain MF-43 (U35255).

■ **Similarity plots.** These were created using Stuart Ray's Simplot 2.5 (Lole *et al.*, 1999). The window size was 200 bp and the step size 20 bp. Jukes-Cantor corrections were applied. S segment sequences of different PUUV strains were used as reference sequences or alternatively, consensus sequences of PUUV lineages were used. The query sequence was either one of the Danish sequences or a consensus query of the three Danish S segment sequences. Phylogenetic trees with 500 bootstrap replicates were calculated on regions showing highest similarities using programs from the PHYLIP package (Seqboot, Dnadist, Neighbour-joining and Consense).

Results

Screening of rodent samples

Altogether, 90 bank vole tissue samples from Karelia were screened for the presence of PUUV N Ag. Positive bank voles were found from all three locations (Table 1). The proportion of the N Ag-positive samples varied between locations from 8% to 25% (Table 1). Three positive samples (one from each location) were selected for RT-PCR, and amplicons of full-length S segment and partial M segment were prepared. Of 152 Danish *C. glareolus* samples eight (four from Fyn, and four from Jutland) were found to be Ab-positive (Table 1). RT-PCR amplicons corresponding to the complete S segment sequence and the partial (nt 2168–2569) M segment sequence were recovered from only one sample in this collection. Later, two more Ab-positive Danish samples from 69 bank voles collected in Fyn in January 2000 (details will be published elsewhere) were added to our analyses and the S and M sequences were recovered from each of them. Six wt PUUV strains, three from

Karelia and three from Denmark, were designated as Puumala/Karhumäki/Cg117/95 (or Karhu117, for short), Puumala/Gomselga/Cg4/95 (Gom4), Puumala/Kolodozero/Cg53/95 (Kolod53), Puumala/Fyn/Cg19/90 (Fyn19), Puumala/Fyn/Cg47/00 (Fyn47) and Puumala/Fyn/Cg131/00 (Fyn131).

Genetic analysis of the Karelian and Danish strains

The S segments of the wt strains from Karelia were 1828–1832 nt in length and strains from Denmark were 1832–1858 nt in length (22 nucleotides from each terminus were involved in primer annealing and therefore were not determined directly). Each carried a single open reading frame of 433 aa for the N protein. In the deduced aa sequences of the N protein Karelian strain markers typical of Finnish strains (Met₂₆₂, Asp₃₀₉ and Phe₃₈₈), but not those typical of Russian (Arg₂₆₇ and Thr₂₇₂), southern Scandinavian (S-SCA) (Asp₉ and Ala₂₀) or northern Scandinavian (N-SCA) (Asp₂₇₂), were observed (Fig. 2A). Similarly, in the deduced aa sequence of the G2 protein (aa 550–683 of the glycoprotein precursor) there is a marker (Val₆₀₃) shared with Karelian and Finnish strains (Fig. 2B). Furthermore, the pattern of insertions/deletions in the 3'-noncoding region (NCR) of the Karelian strains resembled that of Finnish strains (data not shown). These features suggest a connection between these two groups of PUUV strains, which is discussed later.

As for the Danish strains, they seem not to share any pronounced markers with members of other PUUV lineages (Fig. 2). In the deduced aa sequence of the G2 (aa 550–683 of the glycoprotein precursor) one marker specific for the Fyn strains was observed (Leu₅₅₇). Another marker, Asn₆₆₇, is shared by the S-SCA group and the Danish strains. In the deduced aa sequence of the N protein three Danish strains contain distinct aa residues Asp and Ala at positions 272 and 305, respectively, while the S-SCA aa markers at positions 9 and 20 were not found in the sequences of the Danish strains. In some versions of alignments of the S segment 3'NCR, several markers that are shared by the S-SCA lineage and the Danish strains can be selected [e.g. a hexanucleotide deletion at position 1360–1366, of a multiple sequence alignment of 1950 nt, and heptanucleotide AUACA(U/C)(C/U) at position 1687–1694]. However, these markers were not observed in all the versions of the alignment and thus should not be considered indisputable.

Pairwise comparison of PUUV sequences

These studies revealed that the Karelian strains showed highest similarity to Finnish strains (for supplementary data see <http://vir.sgmjournals.org>). For the S segment nt and N protein aa sequences the corresponding values were 91–93% and 96–99%. Other groups/lineages of PUUV strains showed nt identity of 75–84% and aa identity of 92–97%, the strains from Russia being most closely related to the Finnish and

Table 1. Bank vole samples from Karelia and Denmark

Locations	No. of samples	No. of Ag/Ab-positive (%)
Karelia		
Gomselga	4	1 (25)
Kolodozero	61	9 (15)
Karhumäki	25	2 (8)
Total ...	90	12 (13)
Denmark		
Sealand	34	0
Fyn	19	4 (21)
Jutland	99	4 (4)
Total ...	152	8 (5)

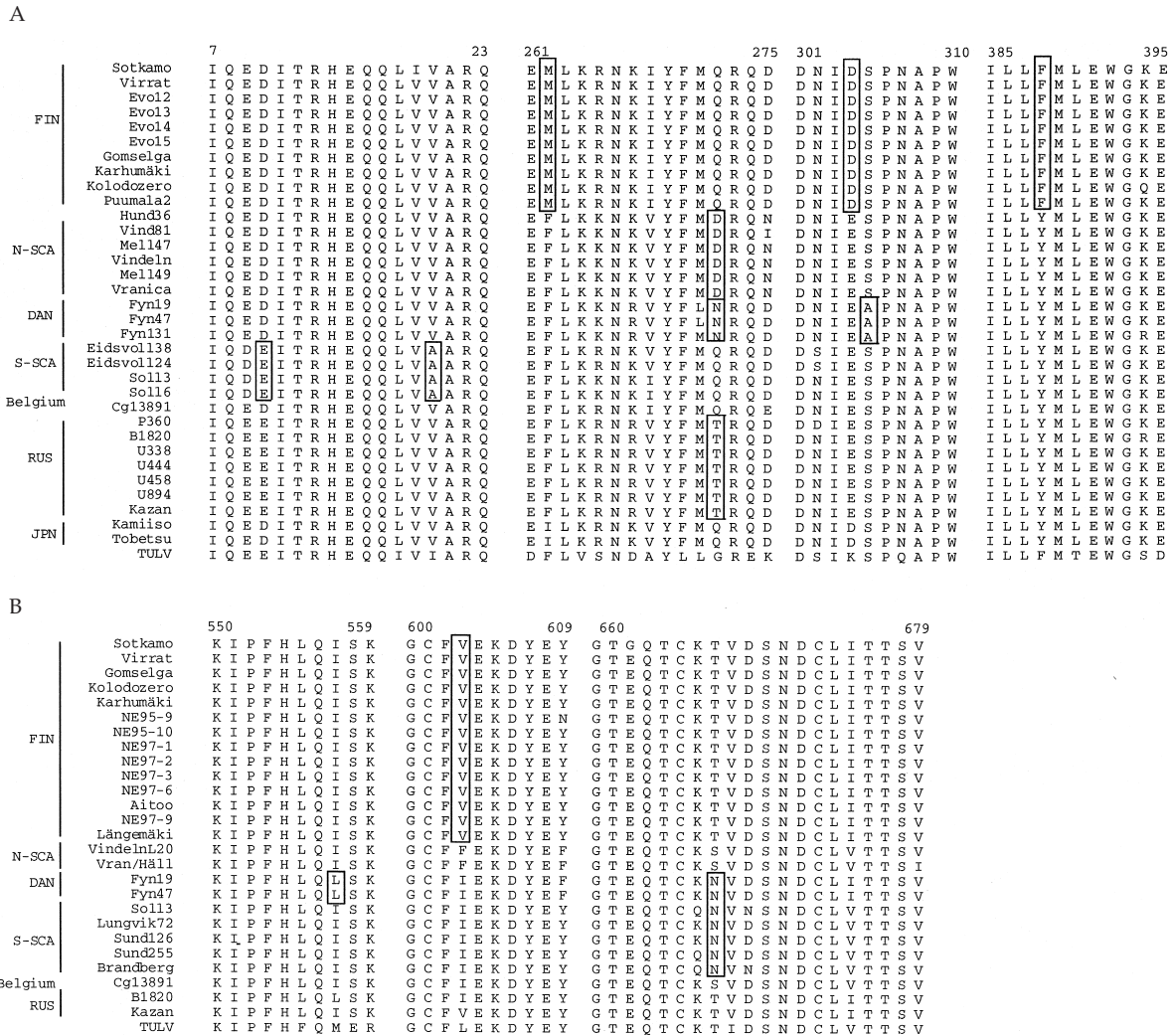


Fig. 2. Amino acid markers in Puumala virus nucleocapsid protein (A) and partial sequence of the glycoprotein G2 (B).

Karelian groups. The same was true for the M segment nt and G2 aa sequences, similarities between Karelian and Finnish strains were 89–94% and 97–100%. Similarly, other groups/lineages had lower identities to Karelian strains than to Finnish strains, and again, the Russian strains were most closely related. This is in line with observations on common sequence markers shared by the Karelian and Finnish groups of strains.

The Danish strains did not show a particularly close relatedness to any of the other PUUV groups: the highest nt identity observed with the Russian group was 78.2% (S segment) or 84.3% (M segment), suggesting a weaker evolutionary connection of the Fyn strains to other PUUV strains.

Phylogenetic analysis

The phylogenetic trees calculated for the complete coding region of the S segment (Fig. 3A) and partial M segment

sequences (Fig. 3B) showed that PUUV strains form seven lineages: Finnish (FIN), S-SCA, N-SCA, Russian (RUS), Belgian (BEL), Japanese (JPN) and Danish (DAN), overall showing a typical geographical clustering of genetic variants. In agreement with the sequence comparison data (for supplementary data see <http://vir.sgmjournals.org>), Karelian strains were placed within the FIN lineage with high bootstrap support while Danish strains formed a distinct genetic lineage whatever algorithm [distant matrix (Fig. 3), parsimony or maximum likelihood (not shown)] was used to infer phylogenies from the nucleotide sequences. The same was seen for the phylogenetic trees calculated on the basis of N or G2 aa sequences (not shown).

To gain more insight into the evolutionary connections of the Danish strains, Simplot analysis of the complete S segment sequences was performed (Fig. 4). None of the PUUV lineages showed higher similarity to the Danish strains than did other lineages; however, the plot revealed two regions in the S

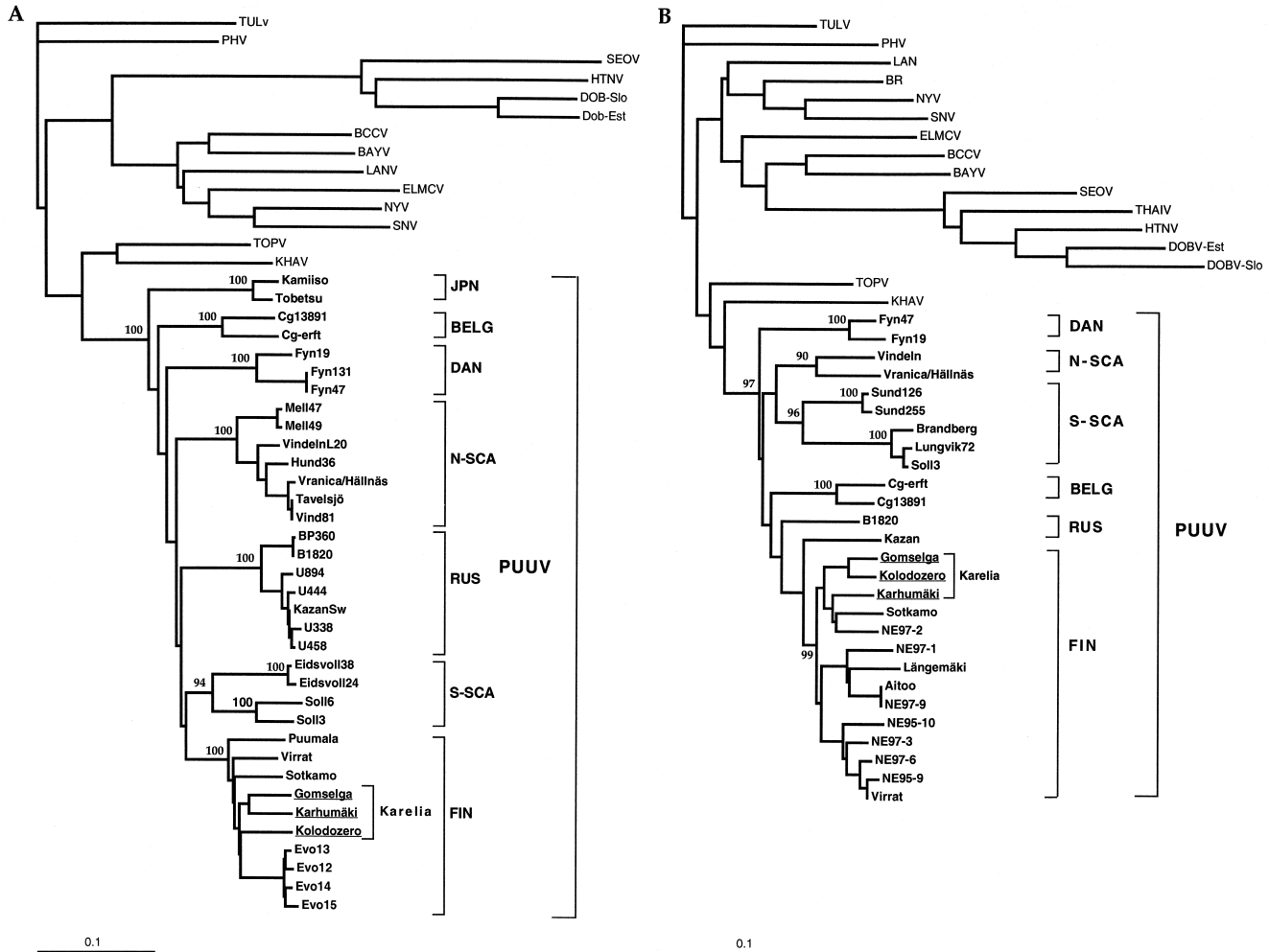


Fig. 3. Phylogenetic trees calculated from the complete coding sequence of the S segment (A) and partial sequence (nt 2168–2569) of the M segment (B) using the Fitch–Margoliash algorithm. Numbers show the bootstrap support values calculated for 500 replicates.

segment with higher similarity of the Danish strains to the N-SCA lineage (nt 650–850) and to the S-SCA lineage (nt 1050–1250, Fig. 4A). Phylogenetic trees were calculated for these regions using the Neighbour-joining algorithm. On the tree calculated based on nt 650–850, the Danish strains were positioned within the N-SCA lineage, and this received substantial bootstrap support (74%, Fig. 4B). On the other tree, placing the Danish strains within the S-SCA lineage was not well-supported (22%, the tree is not shown). When the second region (nt 1050–1250) was studied in greater detail it appeared that the highest peak on the Simplot curve was obtained using Danish strain Fyn19 as a query and the S-SCA lineage split into two sublineages, Eidsvoll and Sollefteå. Furthermore, the corresponding phylogenetic tree placing strain Fyn19 together with the Eidsvoll strain received higher bootstrap support (60%, Fig. 4D).

Thus, based on these data we conclude that the Danish PUUV strains have something in common with both N-SCA

and S-SCA lineages. However, such connections are not indisputable and need to be investigated further.

Discussion

The aim of this work was to study the relationships of PUUV lineages in Fennoscandia using newly characterized strains from two postulated routes of recolonization of these territories by the natural host of the virus, the bank vole, 8000–12000 years ago. Based on geographical proximity, we expected to see Karelian PUUV strains to be related to either Finnish or N-SCA lineages, and Danish strains to be related to the S-SCA lineage. Our analyses of the PUUV strains from Russian Karelia do indeed show that they are most closely related to strains from Finland and share with them a common ancestor. Such grouping received a high bootstrap support, whatever viral sequences were selected for the analyses and whatever algorithm was used to infer phylogenies. This

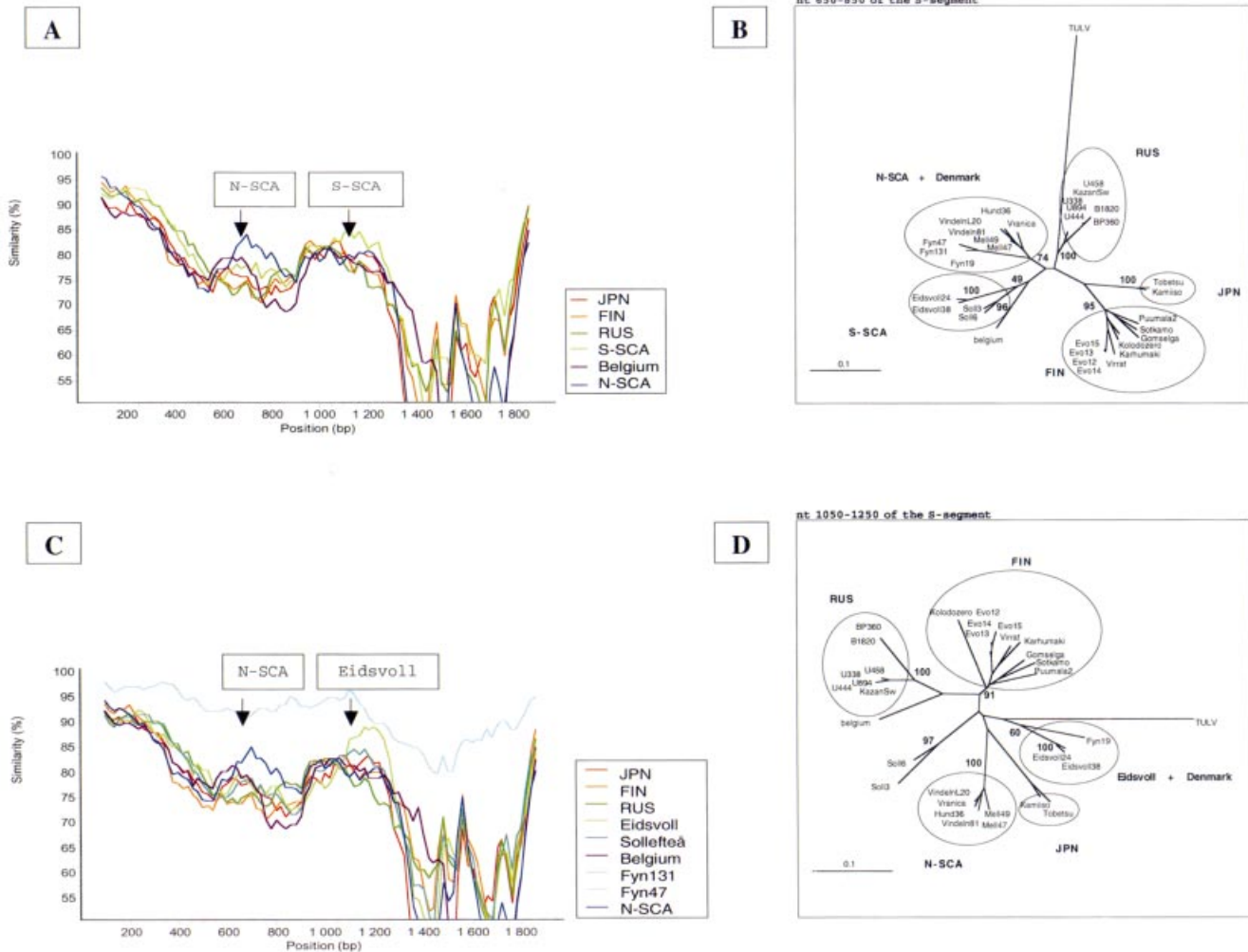


Fig. 4. Similarity plots of the full-length S segment sequences of PUUV and phylogenetic trees calculated for two selected regions. Numbers show the bootstrap support values calculated for 500 replicates. (A) Similarity plot calculated using a consensus sequence of three Danish strains as a query and consensus sequences of different PUUV lineages as references. (B) Neighbour-joining tree based on nt 650–850. (C) Similarity plot calculated using strain Fyn19 as a query and consensus sequences of different PUUV lineages as references (S-SCA lineage was divided into two sublineages, Eidsvoll and Sollefteå). (D) Neighbour-joining tree based on nt 1050–1250.

suggests that the entire area, which includes southern and central Finland and Russian Karelia, was recolonized by the same stream of post-glacial bank vole migrants. On the other hand, as no relatives of members of the N-SCA lineage were found among the Karelian strains, the origin for this lineage remains unknown. At least two possibilities should be considered: (i) PUUV strains of this lineage arrived with bank vole migrants through more northern territories, e.g. along a dry coastal corridor from Kanin to the Kola Peninsula when the sea level was much lower than nowadays; (ii) a tiny land area in northern Norway, which remained uncovered by ice during the last ice-age (Ignatius *et al.*, 1981), could have supported yet another bank vole refugium. Furthermore, it cannot be completely ruled out that ancestor(s) of the N-SCA lineage may have been out-competed from the original migration

route by strains with a higher fitness originating from a different refugium area.

The Danish PUUV strains showed no particularly close similarity to any of the known PUUV strains and formed a distinct phylogenetic lineage on trees calculated for both S and M segment sequences. Thus, no direct link between the Danish PUUV strains and those of the S-SCA lineage was found, suggesting that in this case, too, ancestor(s) of the lineage may have been extirpated from the migration route by more successful variant(s).

Interestingly, within the S segment of Danish PUUV strains we have found two regions with higher than average similarity to members of N-SCA or, to a lesser extent, S-SCA genetic lineages (Fig. 4). An earlier study (Hörling *et al.*, 1996) has clearly shown that the N-SCA and S-SCA lineages of PUUV

are associated with two distinct bank vole populations. This supports a bi-directional scenario of PUUV spreading into Fennoscandia with bank voles from different glacial refugia populations that had met in central Sweden to form a narrow contact zone. However, some recent findings indicate that the phylogeographical pattern of the bank vole is perhaps not that simple, the Danish population belonging to the north-eastern lineage and not to the southern lineage (Jaarola *et al.*, 1999). The issue has been complicated even more by the finding of 'southern' and reassortant types of PUUV in the north-east lineage of bank voles in Russia (Morzunov *et al.*, 1999). Taking into consideration these new data, one can hypothesize that the sequence similarities observed between the Danish PUUV strains on the one side, and N-SCA or S-SCA strains on the other, reflect the evolutionary relationships of their direct precursor(s). Our findings of a mosaic-like structure of the S segment of the Danish lineage may be interpreted as an indication of recombination event(s) that occurred between precursors of these three lineages. Another possible reason for these dissimilarities might be that different portions of the genome of a common ancestor have been selectively preserved, depending on a different genetic background (created via genetic drift of the virus), in such a way that one portion of the genome had happened to be preserved better in lineages 'A' and 'B', another in lineages 'B' and 'C', etc.

In general, the phylogeny of known PUUV genetic lineages from Fennoscandia, as well as those from Russia, Belgium and Japan, looks 'star-like' (all lineages are radiated from the single spot); thus their more intimate evolutionary relationships still remain obscure and await further investigation. Analyses of PUUV strains in postulated areas of glacial refugia in southern Europe will be crucial in further understanding these evolutionary relationships.

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