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GENETIC CHARACTERIZATION OF PUUMALA HANTAVIRUS STRAINS FROM BELGIUM: EVIDENCE FOR A DISTINCT PHYLOGENETIC LINEAGE

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ABSTRACT

Puumala hantavirus (PUUV) sequences were recovered from red bank voles (*Clethrionomys glareolus*) trapped between 1996 and 1998 in four localities of southern Belgium: Thuin, Montbliart, Momignies and Couvin. In addition, three PUUV isolates originating from bank voles trapped in the 1980s in southern (Montbliart) and northern (Turnhout) Belgium were genetically characterized. Analysis of the complete S and partial M segment sequences showed that the Belgian PUUV strains constitute a genetic lineage, distinct from other known PUUV lineages from Europe and Japan. This lineage also includes a wild strain (Cg-Erft) originating from a neighbouring area of Germany. Within the Belgian lineage, geographical clustering of genetic variants was observed. In the Montbliart site, the range of diversity between the most temporally distant strains (from 1986 and 1996 – 1998) was higher than between those from 1996 and 1998, suggesting slight genetic drift via accumulation of neutral or quasi-neutral substitutions with time.

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Introduction

Hantaviruses are the etiologic agents of haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus pulmonary syndrome (HPS) in Americas (for review, see Schmaljohn and Hjelle, 1997; Mertz et al., 1998). Hantaviruses are negative-stranded RNA viruses and constitute a separate genus in the Bunyaviridae family. Their genome consists of three segments designated small (S), medium (M) and large (L) encoding respectively, the nucleocapsid protein (N), two envelope glycoproteins G1 and G2 and the RNA-dependent RNA-polymerase (Schmaljohn, 1996). Each hantavirus is carried by one main persistently infected rodent or insectivore vector. Rodent hosts belong to the subfamilies Murinae, Arvicolinae and Sigmodontinae within the family Muridae (Plyusnin et al. 1996b; Schmaljohn and Hjelle, 1997). Murinae rodents are reservoir hosts of Hantaan (HTNV), Seoul (SEOV) and Dobrava (DOBV) viruses which are the causative agents of a severe form of HFRS in Asia and Europe (Antoniadis et al., 1996; Lee, 1996; Lundkvist et al., 1997a; Avsic-Zupanc et al., 1999). Puumala (PUUV), Tula (TULV), Topografov (TOPV) and Khabarovsk (KBRV) viruses are associated with Arvicolinae rodents in Eurasia (Plyusnin et al., 1996a,b). PUUV is carried by the red bank vole (*Clethrionomys glareolus*) and causes a moderate clinical form of HFRS, nephropathia epidemica (NE) (reviewed by Kanerva et al., 1998). Sin Nombre (SNV)-like viruses are responsible for HPS in Americas and are carried by Sigmodontinae rodents (for review, see Mertz et al., 1998; Peters et al., 1999). The geographical distribution of hantavirus infection in humans generally overlaps the biotope of the related rodent host. In northern Europe, NE epidemics occur with a 3–4 year periodicity, following peaks of red bank voles cyclic populations (Brummer-Korvenkontio et al., 1982; Niklasson et al., 1995). Several hundreds of human cases are registered annually in the Scandinavian countries (Mustonen et al., 1998). Minor NE outbreaks have also been reported in France, Germany, The Netherlands and Belgium (Pilaski et al., 1991; Clement et al., 1994; Le Guenno et al., 1994; Groen et al., 1995; Heyman et al., 1999). The two most recent epidemics (1992–1993 and 1995–1996) reported in France and Belgium occurred in a mixed pine and broad leaf forested area located on both sides of the Franco–Belgian border (Clement et al., 1994; Le Guenno et al., 1994; Heyman et al., 1999). Sporadic NE cases are still reported each year in this region. Phylogenetic studies showed that all PUUV strains share a common ancestor and that variants from Scandinavia, Russia, Austria and western Europe constituted distinct lineages within the PUUV group (Plyusnin et al., 1996b; Bowen et al., 1997; Lundkvist et al., 1998; Heiske et al., 1999). A lineage represented by the PUUV-like strains carried by *Clethrionomys rufocanus* has also been described in Hokkaido, Japan (Kariwa et al., 1999). No human infection associated with these variants has been reported and no antibodies in human sera have been found so far. Previous studies have identified the red bank vole as the main reservoir for hantavirus infection in Belgium and, so far, PUUV has been the only hantavirus type incriminated in HFRS disease (Verhagen et al., 1987; Clement et al., 1994; Heyman et al., 1999). A survey undertaken in southern Belgium showed a high seroprevalence of PUUV infection in red bank voles and an apparent widespread distribution of the virus during the fall of the 1996 epidemic year (Escutenaire et al., 2000). Also, a good concordance was observed between serological data and detection of PUUV genome by the reverse transcription-polymerase chain reaction (RT-PCR). To characterize the genetic diversity and the phylogenetic relationships of

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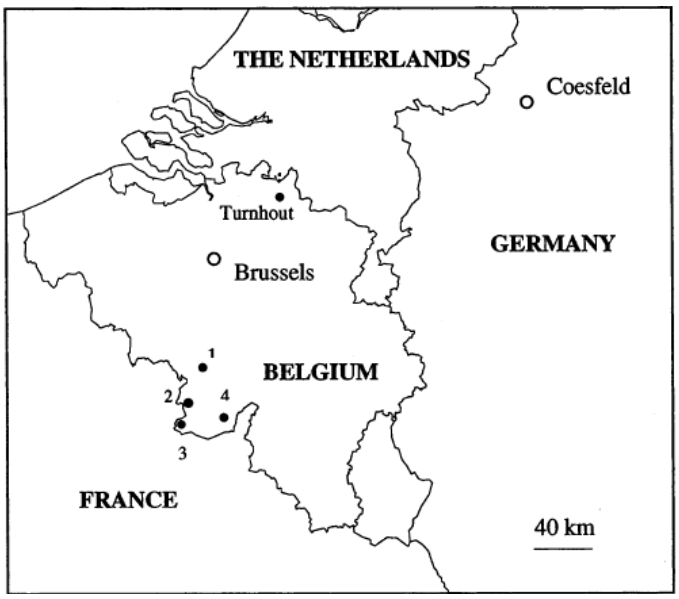
wild PUUV strains circulating in southern Belgium, sequences of the M and S viral RNA segments were recovered from lung tissue samples of PCR-positive rodents trapped in four distinct locations: Thuin, Montbliart, Momignies and Couvin. In addition, M and S segment sequences were recovered from three PUUV cell culture isolates, which originated from bank voles trapped between 1984 and 1986 in Montbliart and in Turnhout, a town located in the northern part of the country (van der Groen et al., 1987).

Materials and methods

TRAPPING OF RODENTS AND SEROLOGICAL SCREENING

From 1996 to 1998, small mammals were trapped twice a year (in fall and spring) in four main localities of the southern NE-epidemic region of Belgium (Fig. 1). Blood samples were collected from the retro-orbital sinus and sera were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of hantavirus-specific IgG antibodies as described previously (Escutenaire et al., 2000). Briefly, PUUV (strain Cg18-20; Tkachenko et al., 1984), HTNV (strain 76-118; Lee et al., 1978) and DOBV (strain Dobrava; Avsic-Zupanc et al., 1992) infected Vero E6 cell lysates were adsorbed to microtitre plates at 4°C overnight. Serum samples, diluted 1:100, were incubated for 1 h at 37°C. Specific antibody binding was detected by peroxidase-labelled goat anti-mouse IgG (Sigma), diluted 1:5000. TMB substrate (Sigma) was added after 1h at 37°C and the reaction was stopped after 10 min with 1 N H_2SO_4. Non infected Vero E6 cell lysates were used as a negative control. The optical density was determined on both viral and negative control antigen at 450 nm and the cut-off value was estimated by a log-likelihood ratio method (Parker et al., 1990; Vizard et al., 1990).

Fig. 1. *Geographical distribution of the trapping localities (•) in Belgium. The town of origin of the German Puumala strain Cg-Erft (Coesfeld) is also marked. 1: Thuin; 2: Montbliart; 3: Momignies; 4: Couvin.*



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PCR AMPLIFICATION AND SEQUENCING

For the detection of viral RNA, a total of 180 red bank voles were tested by RT-PCR. RNA from ground lungs was extracted by the acid guanidium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). The cDNA was amplified by a nested-PCR strategy using S genus-specific and M PUUV-specific oligonucleotides (Escutenaire et al., 2000). The expected size of the amplified fragments was 205 base pairs (bp) [nucleotide (nt) 1033–1237] and 310 bp (nt 2463–2772) for the S and the M segments, respectively. The PCR products were sequenced by the dideoxy method (Sanger et al., 1977) with both inner primers for the nested-PCRs.

To generate longer sequences from specimens of each trapping locality, three additional PCRs were used. The complete S segment was amplified according to RT-PCR conditions previously described (Plyusnin et al., 1994). PCR products were purified with the QIAquick kit (QIAgen) and cloned with the pGEM-T Cloning kit (Promega). Plasmids were purified with the QIAprep kit (QIAgen) and sequenced automatically with M13F and M13R Dye Primer sequencing kits (Perkin–Elmer). Fragments of 592 bp (nt 3090–3681) and 538 bp (nt 1310–1847) of the 3' part of the M and S segments, respectively, were also amplified and automatically sequenced. In addition, for three strains, longer fragments of the M segment (nt 2484 – 3681) were generated and sequenced for bootscanning analysis.

BELGIAN VERO E6 ISOLATES

S and M segment sequences were determined for three Belgian PUUV Vero E6 isolates: CG13891 originated from bank voles trapped in the northern town of Turnhout in 1984 (van der Groen et al., 1987), and CG14444 and CG14445 from bank voles trapped in Montbliart in 1986 (van der Groen and Verhagen, unpublished data).

The complete S segment was amplified, cloned and sequenced as described above. The sequence of the complete M segment of the isolate CG13891 was amplified in three overlapping parts that included nt 1–1177, 992–2483 and 2371–3681. A part of the M segment (nt 2463–2772) from the isolate CG14444 was also amplified and sequenced.

PHYLOGENETIC ANALYSIS

Nucleotide sequences were analysed using the PHYLIP program package (Felsenstein, 1993). Two thousand bootstrap replicates of the sequence data were generated by Seqboot. Distance matrices were calculated using Kimura's two-parameter model (Dnadist) with a transition/transversion ratio of 2.0 and introduced in the Fitch-Margoliash (FM) tree fitting algorithms. The occurrence ratios of branchings were calculated from the trees by Consense. Bootscanning (Salminen et al., 1995, 1997) of partial M sequences (nt 2484–3681) was performed with the BootScan program from the SimPlot package[2] (Ray, 1999). Bootscanning involved 500 bootstrap replicates, distance calculation by Kimura's two-parameter model (Dnadist) with a transition/transversion ratio of 2.0 and tree construction by the FM algorithm for a window of 200 nt, with 20-nt step.

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NUCLEOTIDE ACCESSION NUMBERS

The sequences of the complete S segment of PUUV strains CG14444, CG14445, Thuin/33Cg/ 96, Montbliart/23Cg/96, Momignies/47Cg/96, Momignies/55Cg/96 and Couvin/59Cg/97 have been assigned EMBL accession no. AJ277075, AJ277076, AJ277030, AJ277031, AJ277032, AJ277033 and AJ277034, respectively. The S segment 3' non coding region (NCR) sequences of PUUV strains Momignies/18Cg/98, Momignies/7Cg/98, Montbliart/15Cg/98, Montbliart/18Cg/98 and Montbliart/33Cg/97 were deposited under EMBL accession no. AJ277035, AJ277036, AJ277037, AJ277038 and AJ277039, respectively. The EMBL accession no of the partial M segment sequences (nt 2484–3681) of the PUUV strains Couvin/59Cg/97, Momignies/47Cg/96 and Montbliart/33Cg/97 are AJ277040, AJ277041 and AJ277042, respectively. The partial M segment sequences (nt 3133–3681) of PUUV strains Montbliart/15Cg/98, Thuin/23Cg/96 and Montbliart/18Cg/98 have been assigned EMBL accession no AJ277043, AJ277044 and AJ277045, respectively.

Results

RT-PCRS ON RODENT SAMPLES

Both partial S and M segment sequences (Table 1) were recovered by RT-PCR from 21 out of 180 red bank voles trapped between 1996 and 1998 in four main localities of southern Belgium (Fig. 1). For 13 of them, a serum sample was also available: 11 were positive for PUUV antibodies by ELISA and two were negative. Abbreviations for the wild PUUV strains are presented in Table 1.

GENETIC ANALYSIS

Sequences of the complete S and M segments of CG13891 were 99.6 and 100% nt similar, respectively, with the corresponding sequences of the strain referred as PUU90-13, isolated in France in the early 1990s (Bowen et al., 1995; Rollin et al., 1995). These results and the former handling of the isolate CG13891 in the laboratory where the PUU 90-13 strain was isolated have suggested that the published sequence was in fact that of the Belgian virus; the name PUU90-13 has therefore been withdrawn (Rollin et al., 1999).

COMPLETE S SEGMENT SEQUENCES

The S segment sequence from the three Vero E6 isolates and five wild strains consisted of 1837 – 1840 nt and contained an open reading frame (ORF) coding for 433 amino acids (aa) of the nucleocapsid protein (N). The 5'and 3'NCRs were 42 nt and 493 – 496 nt in length, respectively.

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Table 1. *Puumala virus nucleotide sequences recovered from bank voles*

Locality/animal/year of collection	Abbreviations	Nucleotide sequence determined				
		S segment			M segment	
		nt 1033–1237	nt 1345–1837/40[a,b]	complete	nt 2463–2772	nt 3090–3681[a]
Thuin						
PUU/Thuin/23Cg/96	T23	+			+	+
PUU/Thuin/28Cg/96	T28	+			+	
PUU/Thuin/29Cg/96	T29	+			+	
PUU/Thuin/33Cg/96	T33	+		+	+	
Montbliart						
PUU/Montbliart/23Cg/96	Mt23	+		+	+	
PUU/Montbliart/33Cg/97	Mt33	+	+		+	+
PUU/Montbliart/7Cg/98	Mt7	+			+	
PUU/Montbliart/8Cg/98	Mt8	+			+	
PUU/Montbliart/15Cg/98	Mt15	+	+		+	+
PUU/Montbliart/18Cg/98	Mt18	+	+		+	+
PUU/Montbliart/20Cg/98	Mt20	+			+	
Momignies						
PUU/Momignies/2Cg/96	Mo2	+			+	
PUU/Momignies/10Cg/96	Mo10	+			+	
PUU/Momignies/11Cg/96	Mo11	+			+	
PUU/Momignies/41Cg/96	Mo41	+			+	
PUU/Momignies/47Cg/96	Mo47	+		+	+	+
PUU/Momignies/55Cg/96	Mo55	+		+	+	
PUU/Momignies/7Cg/98	Mo7	+	+		+	
PUU/Momignies/18Cg/98	Mo18	+	+		+	
Couvin						
PUU/Couvin/14Cg/96	C14	+			+	
PUU/Couvin/59Cg/97	C59	+		+	+	+

[a] Positive samples used for the genetic analysis.
[b] Complete S segment sequences of the strains from Thuin and Montbliart, Couvin and Momignies were 1837, 1839 and 1840 nt in length, respectively.

A multiple nt alignment of all known complete sequences of the S segment coding region of PUUV was prepared. The N-ORF of all Belgian strains shared 15 common nt substitutions, with respect to other PUUV sequences (the German strain Cg-Erft excepted). Among them, six were located between nt 813 and 888, i.e. within the most variable region of the N-ORF (Vapalahti et al., 1992; Plyusnin et al., 1994). The sequence of the strain CG13891 differed from those of the strains from southern Belgium by seven nt; three of them being located between nt 792 and nt 894. Among the 15 substitutions common to the Belgian group, 13 were also shared by the strain Cg-Erft. The isolate CG13891 differed from this strain by the same seven nt as the strains from southern Belgium. The alignment of the complete S segment sequences, which consisted of 1944 positions, showed that the Belgian strains shared common nt stretches at positions 1471–1474 (C/ TATC) and 1736–1745 (ACTTGTATTT), and common deletions at positions 1475–1476, 1687–1688 and 1692–1694, with respect to the sequences of other PUUV strains. The sequences of viruses originating from the southern epidemic area also presented common specific stretches positions 1598–1604 (AGATTTA) and 1656–1659 (CTC/TA), and common deletions located between positions 1585–1588 and 1751–1756.

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Table 2. *Ranges of nucleotide and amino acid differences (%) between viruses from the four trapping localities and selected Puumala strains*

Ranges of nucleotide and amino acid differences (%) between viruses from the four trapping localities and selected Puumala strains

	Belgium		Germany		Sweden	Russia	Norway	Finland	Japan
	Locality 1–4[a]	CG13891	CG-Erft	Berkel	VindL20	CG18-20	Eid1124	Sotk	Tob
Locality 1–4[a]									
S segment (nt 43–1345)	1.3–3.3	7.0–7.6	5.6–6.4	15.0–15.3[b]	19.1–19.4	18.5–19.2	17.1–18.2	17.5–18.4	19.7–20.7
N protein (aa 1–433)	0–0.9	1.2–1.9	1.2–1.9	1.4–2.3[c]	2.4–2.8	4.3–5.0	2.6–3.3	2.8–3.6	4.3–5.0
M segment (nt 3133–3487)	0.9–4.1	8.3–11.9	6.2–7.1	NA[d]	21.7–23.7	22.1–24.0	23.3–27.2	19.9–22.1	NA
G2 protein (aa 1031–1148)	0–0.9	1.7–2.6	0–0.9	NA	15.4–17.3	7.3–8.1	10.3–12.1	11.3–13.1	NA

[a] Locality 1: Thuin; locality 2: Montbliart; locality 3: Momignies; locality 4: Couvin.
[b] Based on sequence from nt 173 to 1239.
[c] Based on sequence from aa 44 to 399.
[d] NA: not assessed.

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When compared to other PUUV strains, deduced N protein sequences of all Belgian strains and Cg-Erft presented the same non-conservative aa substitution at position 64 (Gln instead of Lys/Arg). Within the Belgian group, nine aa mutations were observed. Among them, five were clustered between positions 257 and 284, located in the most variable part of the N protein. Notably, all strains from the southern epidemic region differed from the isolate CG13891 by two non-conservative substitutions (Ala260 → Thr and Ala305 → Ser). Interestingly, Ala residues were also found at the same positions in the N protein sequence of Cg-Erft. Both strains recovered in Montbliart in 1986 differed from the strain circulating in the same locality in 1996 by two conservative aa mutations (Lys264.Arg and Val326.Ile).

PARTIAL M SEGMENT SEQUENCES (NT 3133 – 3681)

The sequence of the M segment selected for our analysis contained a part of the G2 coding region (nt 3133–3487) and 194 nt of the 3'NCR. Comparison of the Belgian strains with those from other parts of Europe showed seven nt substitutions in the coding region common to the Belgian group and Cg-Erft. The strains from the southern epidemic area also differed from other PUUV strains, including CG13891 and Cg-Erft, by one nt at position 3425. Nine additional substitutions common to PUUV strains from Belgium were observed in the 3'NCR.

The deduced aa sequences between positions 1031 and 1148 of G2 showed a common substitution in all Belgian strains and Cg-Erft (Leu103 instead of Met, Gly or Val in other PUUV strains). The strains from southern Belgium and Cg-Erft also differed from CG13891 by one aa residue (Val1122.Ile)).

GENETIC DIVERSITY AMONG PUUV STRAINS

Data on the comparison of the S/N and M/G2 sequences from strains of southern Belgium with those of other PUUV variants are presented in Table 2. Although our trapping area is more distant from the Nordrhein–Westfalen region than from the Belgian town of Turnhout (Fig. 1), PUUV strains of southern Belgium were more closely related to the German virus Cg-Erft than to CG13891. However, comparison of the S/N sequences showed that the Belgian viruses were more distantly related to Berkel, also originating from western Germany. The nt and aa divergences increased when the viruses from the south of Belgium were compared to other European and Japanese PUUV strains. Comparison of the partial M/G2 sequences showed a diversity slightly higher than that obtained with the S/N sequences. Comparison of sequences between the four trapping localities showed that the genetically closest populations were from Momignies and Couvin. Comparison of the only N-ORF sequences showed the lowest nt divergence (0.2%) between two strains from Momignies. Comparison of the S-3'NCR sequences from the Belgian wild strains revealed a slightly higher variability (Table 3). The two genetically closest populations were, again, from Momignies and Couvin and populations from Thuin and Momignies were the most distantly related. A similar pattern was observed for the M segment region that included nt 2484–2751 (Table 3). Surprisingly, comparison of overlapping sequences of the M segment (nt 2567–2882) of three strains from Montbliart (Mt33), Momignies (Mo47) and Couvin (C59) revealed a lowest genetic difference (1.3%) between the viruses from Montbliart and Momignies. Both other pairs, Mt33/C59 and Mo47/C59, showed a 2.3% nt divergence.

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Table 3. *Nucleotide divergence (%) among wild Puumala strains from the four trapping localities*

	Thuin	Montbliart	Momignies	Couvin
3′NCR of the S segment (nt1345–1825)				
Thuin (1 strain)	NA[a]	3.3–3.5	4.8–5.7	4.6
Montbliart (4 strains)		0–0.2	3.7–4.8	3.5–3.7
Momignies (4 strains)			0.4–0.9	1.7–2.6
Couvin (1 strain)				NA
M segment (nt 2484–2751)				
Thuin (3 strains)	0	2.7–3.1	3.1–3.4	3.1
Montbliart (7 strains)		0–0.4	1.9–2.7	1.9–2.3
Momignies (8 strains)			0–0.8	1.5–1.9
Couvin (1 strain)				NA

[a] NA: not assessed.

The strains CG14444 and CG14445, isolated from Montbliart in 1986, differed for the S/N sequences by 0.3% at the nt level and by 0.2% at the aa level. Comparison of these viruses with the wild strain originating from the same site with a 10-year interval showed genetic divergences of 0.8 and 1.1%, respectively, corresponding to aa differences of 0.5 and 0.7%, respectively. Comparison of the partial M segment sequences that included nt 2484–2751 of CG14444 with those of the strains recovered between 1996 and 1998 showed diversity values 0.8–1.1% at the nt level while wild viruses differed by 0–0.4%. Analysis of the corresponding aa sequences showed that all nt mutations observed were silent. This reflects a slight genetic drift via accumulation of neutral or quasi-neutral nt substitutions with time.

PHYLOGENETIC ANALYSIS

The phylogenetic analysis based on the N-ORF sequences showed that the strains from Belgium and the German strain Cg-Erft constitute within the PUUV group a distinct lineage, well-separated from other lineages formed by the Russian, Scandinavian and Japanese strains (Fig. 2a). Within the Belgo–German lineage, the variants from southern Belgium and Cg-Erft formed a well-supported (99%) sublineage, separated from the northern Belgian strain CG13891. Strains from the localities of Momignies and Couvin were shown to share a common more recent ancestor. Strains CG14444 and CG14445 were clustered together with the wild strain of the same geographical origin, Montbliart.

Phylogenetic trees based on sequences including nt 3133–3487 (Fig. 2b) and nt 2484–2697 (data not shown) of the M segment displayed a similar branching order for the Belgian strains with the strain from Momignies (Mo47) clustering together with the one from Couvin (C59) (bootstrap support of 97%, Fig. 2b). In contrast, in the tree based on sequences of another part of the M segment (nt 2567–2882), the strain Mo47 clustered together with Mt33, albeit with lower bootstrap support (71%, Fig. 2c). Although branch lengths within the group Mo47 – Mt33 – C59 on both M-trees were rather short, the contradictory clustering of the strain Mo47 indicated a possible recombination origin of its M segment. The above-mentioned results of the pairwise comparisons supported such a hypothesis.

To test the assumption of a recombination event, longer fragments of the M segment were generated for the strains Mo47, Mt33 and C59, and bootscanning of the obtained sequences (nt 2484–3681) was performed. Bootstrap support values were calculated for a window of 200 nt

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with 20-nt step using the FM algorithm to infer phylogenies. In the regions including nt 2484–2650 and nt 3250–3681, Mo47 clustered together with C59 with bootstrap supports higher than 90% while in the region located between nt 2750–3150, two peaks with bootstrap values of 77 and 70%, respectively, grouped Mo47 with Mt33 (data not shown). In the part located between these two peaks, i.e. between nt 2850–3000, the relationships between the strains were not resolved. Thus, the bootscan results are not contradictory to the hypothesis on the recombination origin of Mo47. However, it seems that the issue needs to be scrutinized more, taking into account the very short branch lengths on phylogenetic trees calculated for the abovementioned regions of the M segment.

Fig. 2. *Fitch-Margoliash (FM) consensus tree based on complete coding region sequences of the S segment (a). FM phylogenetic trees based on partial sequences of the M segment including nt 3133 – 3487 (b) and nt 2567 – 2882 (c). The bootstrap values greater than 70% are indicated at the branch points. Abbreviations for Puumala virus strains: CG13891, PUU/CG13891; Cg-Erft; PUU/Cg-Erft; T33, PUU/Thuin/33Cg/96; Mt23, PUU/Montbliart/23Cg/96; Mt33, PUU/Montbliart/33Cg/97; CG14445, PUU/ Montbliart/14445Cg/86; CG14444, PUU/Montbliart/14444Cg/86; C59, PUU/Couvin/59Cg/97; Mo55, PUU/Momignies/55Cg/96; Mo47, PUU/Momignies/47Cg/96; Puumala, Puumala/1324Cg/79; Sotk, Sotkamo; Virrat, Virrat/25Cg/95; Evo 15, Evo/15Cg/93; B-P360, Bashkiria/P360; B-1820, Bashkiria/CG1820; Udm894, Udmurtia/894Cg/91; Udm458, Udmurtia/458Cg/88; Udm444, Udmurtia/444Cg/88; VindL20, Vindeln/L20Cg/83; Hund36, Hundberget/36Cg/94; Tav81, Tavelsjö /81Cg/94; Vran/Häll, «Vranica» (presumably Hallnäs B1); Mell-49, Mellansel/49Cg/94; Mell-47, Mellansel/47Cg/94; Eid1138, Eidsvoll/1138Cg/87; Eid1124, Eidsvoll/1124v; Soll-6, Solleftea°/6Cg/95; Soll-3, Solleftea/3Cg/94; Tob, Tobetsu/60Cr/93; Kam, Kamiiso-8Cr-95. Abbreviations for other hantaviruses: HTN, Hantaan virus, strain 76-118; DOB, Dobrava virus; SEO, Seoul virus, strain SR-11; TOP, Topografov virus, strain Ls136V; KBR, Khabarovsk virus, strain MF-43; ILV, Isla Vista virus, strain MC-SB-1; PH, Prospect Hill virus, strain PH-1; TUL76, Tula, strain Tula/76Ma/87; TULv, Tula, strain Moravia/5302v/95; BCC, Black Creek Canal virus; BAY, Bayou virus, strain Louisiana, AND, Andes virus, strain AH-1; NY, New York virus, strain RI-1; SN, Sin Nombre virus, strain NM H10; RIOS, Rio Segundo virus, strain RMx-Costa-1; ELMC, El Moro Canyon virus, strain RM-97. NPB and SPB: north and south of the bank vole population border, respectively. Genbank accession numbers. The S segment sequences included PUU, strain PUU/CG13891 (U22423); PUU, strain PUU/Cg-Erft (AJ238779); PUU, strain Puumala/1324Cg/79 (Z46942); PUU, strain Sotkamo (X61035); PUU, strain Virrat/25Cg/95 (Z69985); PUU, strain Evo/15Cg/93 (Z30705); PUU, strain Bashkiria/P360 (L11347); PUU, strain Bashkiria/CG1820 (M32750); PUU, strain Udmurtia/894Cg/91 (Z21497); PUU, strain Udmurtia/458Cg/88 (Z30707); PUU, strain Udmurtia/444Cg/88 (Z30706); PUU, strain Vindeln/L20Cg/83 (Z48586); PUU, strain Hundberget/36Cg/94 (AJ223371); PUU, strain Tavelsjo¨ /81Cg/94 (AJ223380); PUU, strain «Vranica»/Ha¨llna¨s (U14137); PUU, strain Mellansel/49Cg/94 (AJ223375); PUU, strain Mellansel/47Cg/94 (AJ223374); PUU, strain Eidsvoll/1138Cg/87 (AJ223369); PUU, strain Eidsvoll/1124v (AJ223368); PUU, strain Solleftea°/6Cg/95 (AJ223377); PUU, strain Solleftea°/3Cg/94 (AJ223376); PUU, strain Tobetsu/60Cr/93 (AB010731); PUU, strain Kamiiso-8Cr-95 (AB010730); HTN, strain 76-118 (M14626); DOB (L41916); SEO, strain SR-11 (M34881); TOP, strain Ls136V (AJ011646); KBR, strain MF-43 (U35255); ILV, strain MC-SB-1 (U31534); PH, strain PH-1 (Z49098); TUL, strain Tula/76Ma/87 (Z30941); TUL, strain Moravia/5302v/95 (Z69991); BCC (L39949); BAY, strain Louisiana (L36929); AND, strain AH-1 (AF004660); NY, strain RI-1 (U09488); SN, strain NM H10 (L25784) and RIOS, strain RMx-Costa-1 (U18100) and ELMC, strain RM-97 (U11427). The M segment sequences included PUU, strain PUU/CG13891 (U22418); PUU, strain PUU/Cg-Erft (AJ238778); PUU, strain Vindeln/L20Cg/83 (Z49214); PUU, strain «Vranica»/Ha¨llna¨s (U14136); PUU, strain Sotkamo (X61034); PUU, Bashkiria/CG1820 (M29979); PUU, Bashkiria/P360 (L08755) and TUL, strain Tula/Moravia/5302v/95 (Z69993).*

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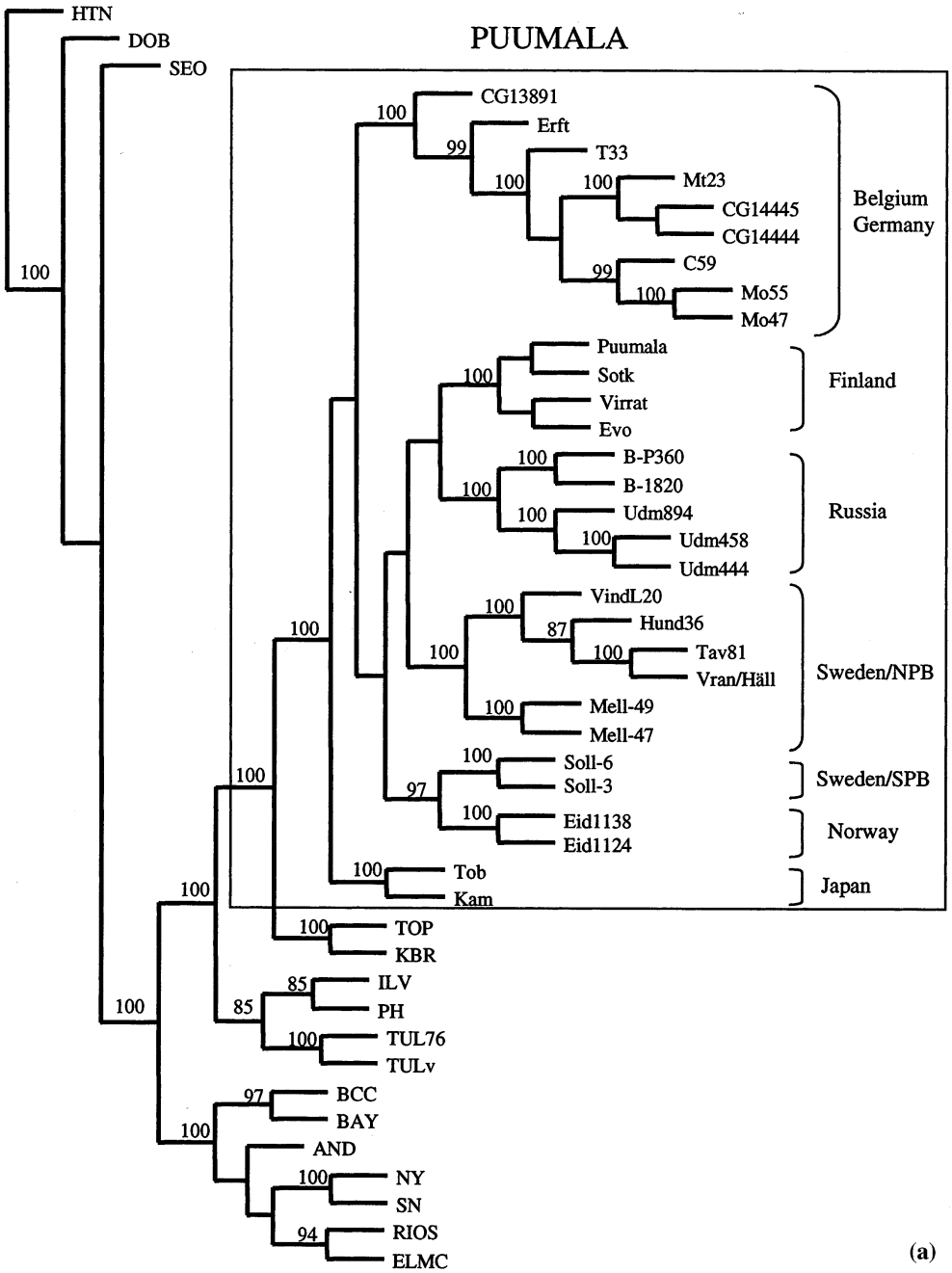

(a)

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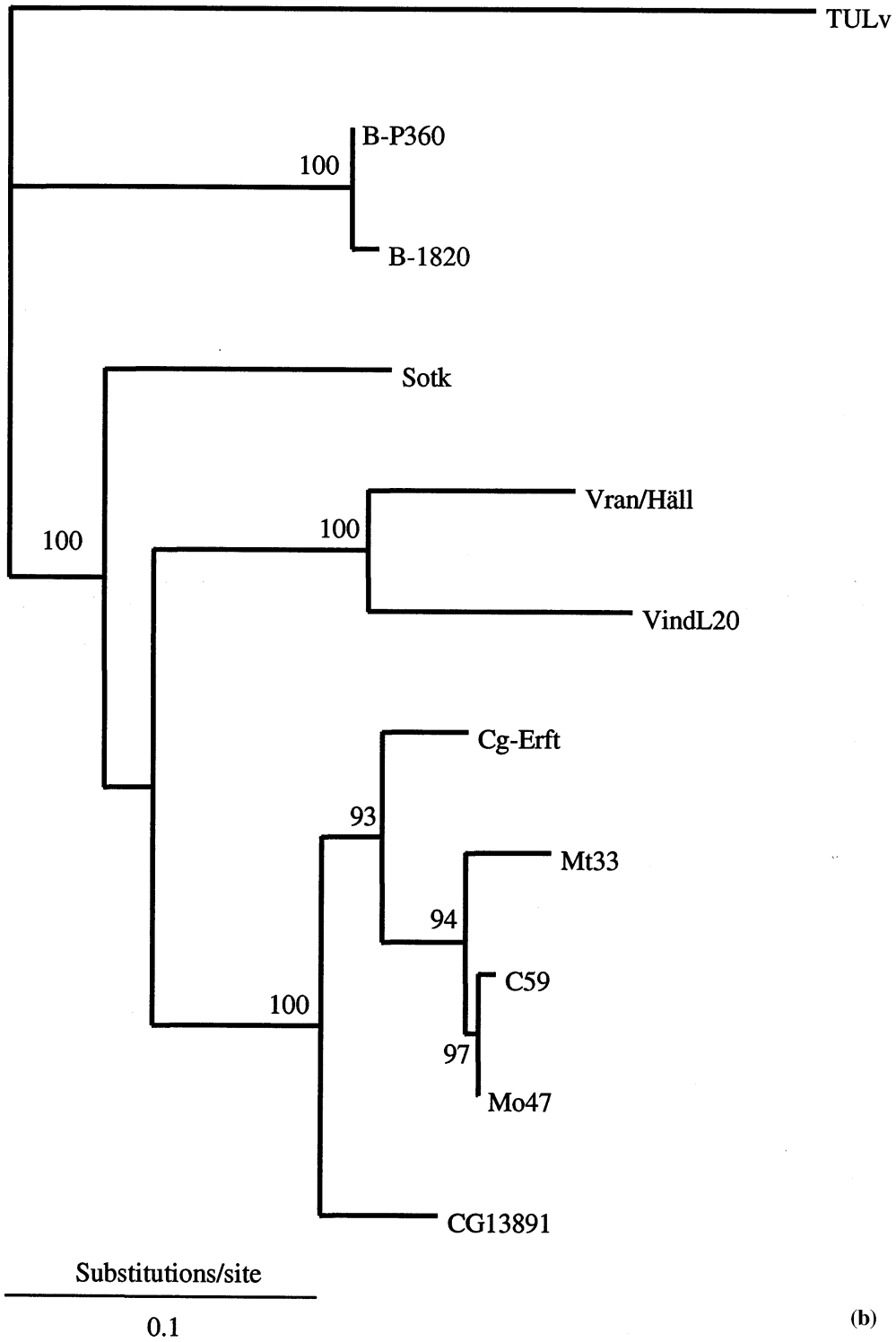
Fig. 2 continued



(b)

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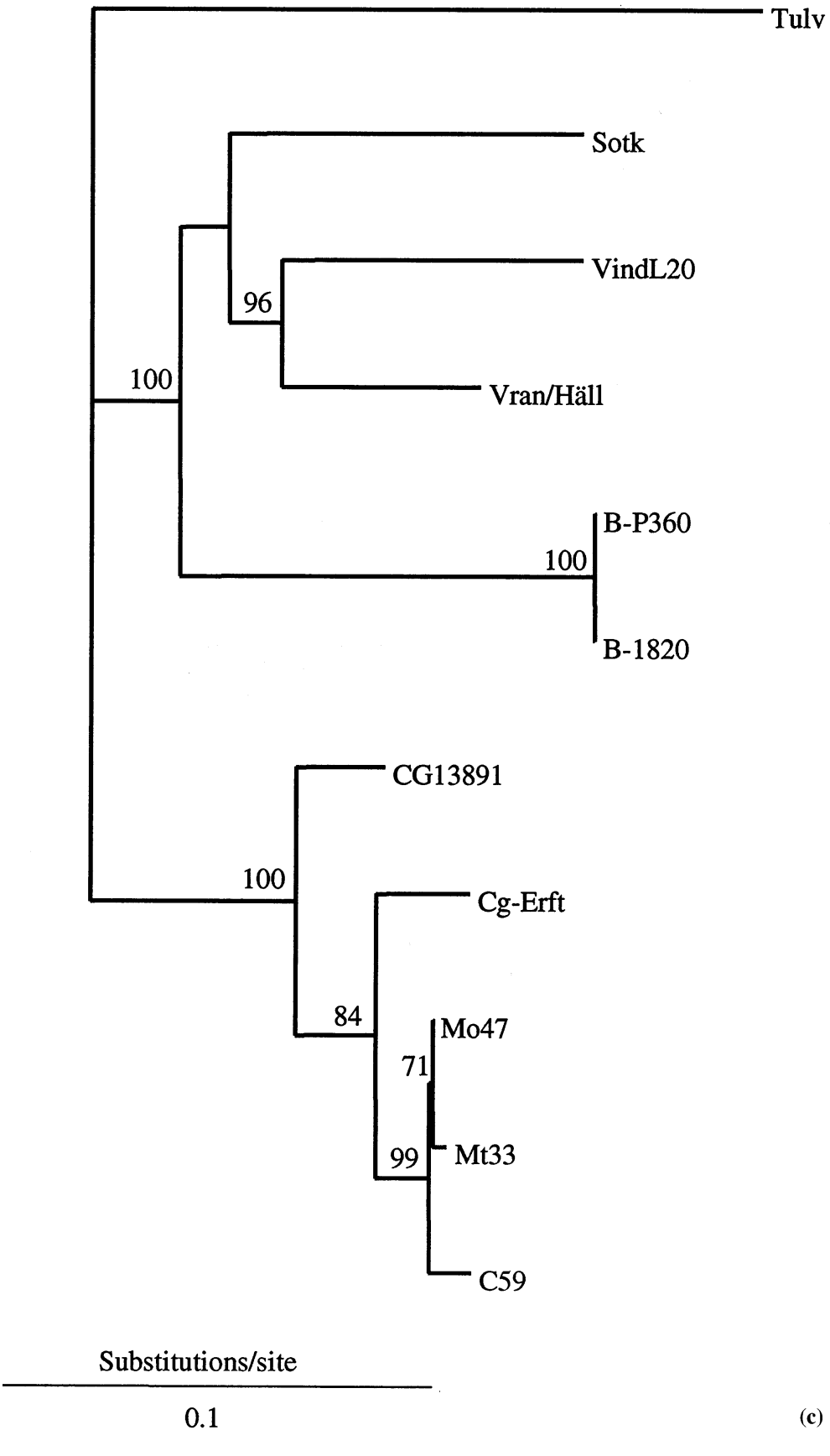
Fig. 2 continued



(c)

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Discussion

GENETIC DIVERSITY OF PUUV STRAINS FROM BELGIUM

With two major outbreaks recorded in 1992–1993 and 1995–1996 and sporadic cases occurring each year, the southern part of Belgium represents an endemic area for NE (Clement et al., 1994; Heyman et al., 1999). Of a total of 180 red bank voles trapped in this region between 1996 and 1998, 21 were found positive for the presence of PUUV RNA. Based on the S and M sequences, the analysis of genetic diversity showed a geographical clustering of the Belgian variants. The strains, originating from localities within a radius of 40 km, were the genetically least divergent with nt diversity values of 1.3–5.7% for the S segment and of 0.9–4.1% for the partial M sequences. A comparable range of nt diversity (0.3–4.6% for the S segment) was observed between Swedish PUUV strains originating from localities within a radius of approximately 30 km (Hörling et al., 1996). A lower range of nt divergence (0.5–0.8%) was found in Austria, among PUUV strains recovered from rodents trapped in areas located about 25 km apart (Bowen et al., 1997). Although the two geographically closest sites were Montbliart and Momignies (about 12 km apart), the two genetically closest populations [for the N-ORF (Fig. 2a) and nt 3133–3487 of the M segment (Fig. 2b)] were those from Momignies and Couvin, separated by 25 km. This discrepancy between the geographical and genetic distances might have resulted from the presence of a physical obstacle like a persisting discontinuity of the wooded area between the two closest localities, thus limiting contacts and spreading of the virus between the respective rodent populations. Clustering of the strains from Momignies (Mo47) and Montbliart (Mt33) for the part of the M segment including nt 2567–2882 (Fig. 2c) raised the question of a putative recombinant origin of Mo47. However, detailed scrutiny of the sequences using similarity plots and bootscanning has not provided strong evidence supporting the recombination hypothesis, leaving the issue unresolved. In our opinion, the strongest obstacle for this was the very close relatedness of the three sequences in question, Mo47, Mt33, and C59 (nt identity 97.7–98.7%). Phylogenetic analysis revealed a distinct cluster of PUUV strains at each trapping site. This characteristic branching pattern corroborates previous observations of clustering of the local genetic variants linked to the small home range of the bank voles (Plyusnin et al., 1995; Hörling et al., 1996). The nt divergence between wild strains originating from the same locality ranged between 0.2–0.3% for the N-ORF, 0–0.9% for the S 3'NCR and 0–0.8% for the partial M segment (nt 2484–2697). Similarly, S segment sequences recovered from local bank vole populations in Finland and Russia showed nt divergence values ranging between 0.7–1.7% and 0.9–2.2%, respectively (Plyusnin et al., 1995). In contrast, a variable partial G1 fragment showed a higher genetic difference (3.6%) between SN viruses originating from rodents trapped in California, within 1 km of one another (Hjelle et al., 1994). PUUV strains from southern Belgium and the Vero E6-adapted CG13891 strain isolated in northern Belgium, in a site located at around 120 km apart from the southern epidemic area, differed at the nt level by 7.0–7.6% for the NORF and by 8.3–11.9% for the partial M segment sequences (nt 3133–3487). These data are also in agreement with previous studies where the genetic diversity values between localities at a distance of 100–200 km ranged, for the S segment, from 7 to 8% in Finland (Plyusnin et al., 1995), from 9 to 11% in Sweden (Hörling et al., 1996), and from 11 to 12% in Norway (Lundkvist et al., 1998). However, a lower genetic distance (2.9% for the

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complete S segment) was observed between the Japanese PUUV strains Kamiiso and Tobetsu, originating from sites located 250 km apart (Kariwa et al., 1999). Comparison of sequences from viruses recovered in Montbliart 10 years apart showed that both strains isolated in 1986 were genetically closer to each other (0.3% nt difference for the N-ORF) than to the strain circulating within the same trapping site in 1996 (0.8–1.1%). Previously, similar or slightly higher divergence was observed for PUUV and SNV strains recovered from rodents several years apart (Nerurkar et al., 1994; Plyusnin et al., 1995; Hörling et al., 1996; Monroe et al., 1999). Such a genetic drift, occurring without any significant diversifying selection in a persistently infected rodent host, might be explained by random sampling and founder effect (Domingo et al., 1995). As transmission rate of hantaviruses is dependent on the fluctuations in population density of their hosts, severe population decline may lead (just by chance) to the establishing of new dominant variants carried by the rodents that survived to the crash. Although passages of the viruses in cell culture might have contributed to the genetic divergence as well, previous studies on PUUV and SNV strains revealed that the adaptation to Vero E6 cells did not alter the viral genome significantly (Chizhikov et al., 1995; Lundkvist et al., 1997b). Notably, only two point mutations were observed in the S segment of PUUV when the wild strain was adapted to the cell culture (Lundkvist et al., 1997b). In our study, most nt mutations accumulated during the 10-year period were silent, and both aa substitutions were conservative. This suggests that stabilising selection is operating in the PUUV evolution which occurs via accumulation of neutral or quasi-neutral substitutions.

A DISTINCT SUBLINEAGE OF PUUV STRAINS FROM WESTERN EUROPE

Genetic analysis indicated that the strains from southern Belgium and the isolate CG13891 from the northern part of the country constitute, together with the German strain Cg-Erft, a distinct lineage of PUUV. Our results corroborate a recent study where the strains Cg-Erft and CG13891 were found to cluster together (Heiske et al., 1999). Surprisingly, the strain Cg-Erft was found to be more closely related to PUUV strains from our trapping area in southern Belgium (distant of about 300 km from the German town of Coesfeld) than to the strain CG13891 (originating from an area distant of about 170 km from Coesfeld) (Fig. 1). Cg-Erft was also more distantly related to another German PUUV strain, Berkel, sequences of which were recovered from a human NE case some 70 km away (Pilaski et al., 1994), than to strains from our trapping area. Similar discordance between the geographical and genetic distances was earlier reported in Sweden where PUUV strains from two different lineages were recovered from sites around 50 km apart (Hörling et al., 1996; Lundkvist et al., 1998). The circulation of genetically distinct viruses in the same geographical area was explained by the co-evolution of each sublineage with a different phylogeographical population of bank voles that recolonized the territory after the last glacial period. Similarly, the co-evolution of PUUV strains from Belgium and the strain Cg-Erft with a subspecies of bank voles, different from those carrying Berkel-like PUUV strain(s), might be the reason for the closer genetic relationship between the strains originating from the most distant sites. Biogeographic factors like past barriers to rodent movement, migrations or founder events could also explain the current diversity and distribution of PUUV in Belgium and Germany.

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