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ORIGINAL ARTICLE

A highly divergent Puumala virus lineage in southern Poland

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Abstract Puumala virus (PUUV) represents one of the most important hantaviruses in Central Europe. Phylogenetic analyses of PUUV strains indicate a strong genetic structuring of this hantavirus. Recently, PUUV sequences were identified in the natural reservoir, the bank vole (*Myodes glareolus*), collected in the northern part of Poland. The objective of this study was to evaluate the presence of PUUV in bank voles from southern Poland. A total of 72 bank voles were trapped in 2009 at six sites in this part of Poland. RT-PCR and IgG-ELISA analyses detected three PUUV positive voles at one trapping site. The PUUV-infected animals were identified by cytochrome b gene analysis to belong to the Carpathian and Eastern evolutionary lineages of bank vole. The novel PUUV S, M and L segment nucleotide sequences showed the closest similarity to sequences of the Russian PUUV lineage from Latvia, but were highly divergent to those previously found in northern Poland, Slovakia and Austria. In conclusion, the detection of a highly divergent PUUV lineage in southern Poland indicates the necessity of further bank vole monitoring in this region allowing rational public health measures to prevent human infections.

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Introduction

Hantaviruses are classified within the family *Bunyaviridae*, genus *Hantavirus*. These enveloped viruses contain three single-stranded RNA segments of negative polarity [1]. Two forms of disease have been distinguished in humans: hantavirus cardiopulmonary syndrome (HCPS) in the Americas and haemorrhagic fever with renal syndrome (HFRS) in Eurasia [2]. HFRS caused by Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV) and Seoul virus (SEOV) was reported to have mortality rates of up to 20%.

Nephropathia epidemica (NE) is a mild to moderate form of HFRS with a mortality rate of <1% that is caused by Puumala virus (PUUV), the most common hantavirus in Europe with the bank vole *Myodes glareolus* acting as the natural reservoir [3]. Bank voles are distributed in almost all parts of Europe except a few regions in Southern and Northern Europe [4]. Human infections with PUUV have been found in a large number of European countries [5]. An oscillation of the recorded number of human cases has been reported for different countries and might be caused by cyclic changes in bank vole populations because of beech mast years [6]. PUUV strains in Central Europe show a strong genetic structuring based on the geographic origin of the reservoir [7, 8].

PUUV contains a small (S) segment of 1,784 to 1,882 nucleotides, a medium (M) segment of 3,682 nucleotides and a large (L) segment of 6,550 nucleotides [9, 10]. The S

segment encodes the nucleocapsid (N) protein and a second overlapping open reading frame (ORF) encodes a putative non-structural protein (NSs) that may play a role in pathogenicity to humans and adaptation of the virus to the reservoir host [3, 11]. The M segment encodes for a glycoprotein precursor that is co-translationally cleaved into the glycoproteins Gn and Gc. The L segment encodes the RNA-dependent RNA polymerase [12].

In 2007, for the first time, human hantavirus infections were reported in Poland affecting the Subcarpathian region [13]. The precise morbidity rate in Poland is unknown, because laboratory diagnosis of human infections is quite rare [14]. In addition, little is known about hantavirus infections in their animal reservoirs. Tula virus (TULV) has been detected in common voles *Microtus arvalis* from Poland and the virus strain Łódź was isolated [15]. Boginia virus was first identified in water shrews *Neomys fodiens* from Poland [16]. Furthermore, a co-circulation of shrew-borne Boginia virus, Seewis virus and mole-borne Nova virus was also demonstrated [17]. Recently, the first molecular evidence of PUUV infections in bank voles was reported in northern Poland [18]; however, most human hantavirus infections in this country have been reported in the Subcarpathian region [13, 14, 19]. Further, a survey of hantavirus reservoirs indicated the presence of PUUV, TULV and DOBV in Subcarpathia [14]. This study aims to assess the relationship between PUUV strains in voles from southern and northern Poland and to test for the potential association of PUUV with different bank vole evolutionary lineages.

Materials and methods

Rodent trapping and dissection

Rodents were captured between August and October 2009 in the southern part of Poland at six trapping sites: forests close to Niepołomice (50°0′N, 20°20′E) and Teleśnica Oszwarowa (49°22′N, 22°32′E), near cities Olkusz (50°19′N, 19°30′E), Katowice (50°15′N, 19°4′E) and Miasteczko Śląskie (50°19′N, 19°30′E) and on the island in Solina Lake (49°21′N, 22°29′E) (Fig. 1). Live traps were placed for three to five days and checked every morning. The captured animals were transported in standard plastic mouse cages in groups of up to four animals of the same sex to the laboratory (Jagiellonian University, Kraków, Poland). Within one to seven days after trapping and arrival in the laboratory, the bank voles were euthanized. The frozen carcasses were transferred in dry ice to the Friedrich-Loeffler-Institut (Greifswald-Insel Riems, Germany). Lung tissue and tail samples were collected according to a standard protocol under biosafety level 3 conditions. Chest cavity fluid was obtained by addition of 1 ml sterile phosphate-buffered saline (PBS). The trapping and dissection of samples from northern Poland have been described previously [18].

Serological investigations

For the serological screening the chest cavity fluid was investigated by an IgG-ELISA using a yeast-expressed nucleocapsid protein of PUUV strain Bavaria [20]. The investigations followed the protocol described in Essbauer et al. [21].

Nucleic acid isolation, hantavirus RT-PCR, cytochrome b PCR and sequence determination

RNA was extracted from the lung tissue of bank voles using Qiazol solution. Screening RT-PCR was performed according to a protocol for S segment sequences [20, 21]. 2.5 µl of RNA were targeted using 10 pmol of the primers PUUV 342F (5′-TAT GGT AAT GTC CTT GAT GT-3′) and PUUV 1102R (5′-GCC ATD ATD GTR TTY CTC AT-3′) and the SuperScriptIII one step RT-PCR kit in a final volume of 25 µl. Following reverse transcription at 50 °C for 45 min and inactivation of reverse transcriptase at 94 °C for 2 min, 40 amplification cycles of denaturation at 94 °C for 30 sec, annealing at 46.2 °C for 30 sec, elongation at 68 °C for 1 min and a final extension at 68 °C for 10 min were performed. For further characterization of the novel PUUV strains, extended parts of the S segment, a part of the M segment and two parts of the L segment were amplified using primers C1 (5′-CCC CCT GAT TGT CCT GGT GTA G-3′), C2 (5′-CCA ACT CCT GAA CCC CAT GC-3′), HanLF1 (5′-ATG TAY GTB AGT GCW GAT GC-3′), HanLR1 (5′-AAC CAD TCW GTY CCR TCA TC-3′), PUULF1 (5′-CAR AAR GGT AAT TGT CAA TCT GG-3′) and PUULR1 (5′-GTA TTT ATA GGC CAT ATC YCT AG-3′) following published protocols [20–23]. In addition, for two bank voles from Mikołajki [18] the L segment and complete coding sequences (CDS) of the S segment were determined. Amplification products were run in 1.5% agarose gels and visualized with ethidium bromide and UV illumination. The evolutionary lineages to which the bank voles belonged were identified by cytochrome b (*cyt b*)-specific PCR following previously described protocols [18, 24]. DNA sequence determination was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Perkin Elmer, Waltham, MA, USA) on an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Sequence comparison and phylogenetic analyses

The best substitution model was determined by jModelTest followed by phylogenetic reconstructions with Bayesian

Fig. 1 Map of Poland showing the trapping sites of bank voles in southern Poland. Puumala virus positive and negative trapping sites are given in red and green, respectively. Black dots represent the sites in northern Poland and Latvia where Puumala virus sequences were detected previously [18, 33]

algorithms via MrBayes v.3.2.2 [25]. The S segment, M segment, L segment and *cyt b* alignments were analysed by the substitution models TIM2+I+G (S, CDS), TrN+G (M), HKY+I+G (L, positions 2956-3366), GTR+G (L, positions 505-1036) and TPM2uf+I (*cyt b*). For Maximum likelihood analyses the substitution models TrN+I+G (S, CDS), TrN+G (M), HKY+I+G (L, positions 2956-3366), GTR+G (L, positions 505-1036) and TrN+I (*cyt b*) were

used. All phylogenetic calculations were performed via the CIPRES online portal [26]. Evolutionary lineages of the bank voles were determined by phylogenetic analyses of partial *cyt b* sequences in comparison to lineage specific sequences obtained from GenBank.

Results

During 2009 a total of 72 bank voles were trapped at six sites in the southern part of Poland (Fig. 1). The serological and RT-PCR screening of all bank voles revealed three positive animals, all originating from the trapping site Teleśnica Oszwarowa (Table 1 and Table 2). One of the animals was anti-PUUV and PUUV-RNA positive, whereas the other two were only serologically or RT-PCR positive.

A comparison of the two partial S segment sequences and the corresponding two N protein sequences (KS14/118, KS14/121) revealed a nucleotide sequence similarity of 98.4% and an amino acid sequence similarity of 98%, respectively (Table S1). Both sequences are highly divergent from the PUUV S segment sequence previously found in northern Poland and sequences of other PUUV clades (Table S1). In phylogenetic analyses, the sequences from southern Poland clustered with sequences of the Russian (RUS) PUUV lineage, but were clearly divergent from sequences of other PUUV clades, including sequences from northern Poland, Slovakia and Austria (data not shown).

For the two RT-PCR positive samples from southern Poland the (almost) complete N protein CDS was determined and showed a nucleotide and amino acid sequence similarity of 97.6% and 98.1% in the CDS and the N protein, respectively (Table 2 and data not shown). The

Table 1 Results of the serological investigation, S segment RT-PCR and cytochrome b-based identification of evolutionary lineage for the bank voles collected at six trapping sites in the southern part of Poland

Trapping site	Number of seropositive animals/total	Number of S segment RT-PCR positive animals/total	Bank vole lineage
Niepołomice	0/12	0/12	Carpathian
Teleśnica Oszwarowa	**2/12**	**2/12**	Eastern, Carpathian[x]
Katowice	0/11	0/11	Carpathian
Solina Lake	0/12	0/12	Eastern, Carpathian
Olkusz	0/15	0/15	Carpathian
Miasteczko Śląskie	0/10	0/10	Carpathian
Total	**2/72**	**2/72**	

[x] for details see Table 2

Table 2 Results of the IgG-ELISA, RT-PCR and cytochrome b-based identification of evolutionary lineage for the 12 bank voles from Teleśnica Oszwarowa, southern Poland

Number	IgG-ELISA	S partial **P355-1065**	S CDS **P43-1344**	M partial **P2393-3010**	L partial **P2956-3366**	L partial **P490-1036**	Bank vole lineage
KS14/118	-	**KX815395**	**KX815395**	**KX815397**	**KX815400**	**KX815403**	**Carpathian**
KS14/119	-	-	n.d.	n.d.	n.d.	n.d.	Eastern
KS14/120	-	-	n.d.	n.d.	n.d.	n.d.	Carpathian
KS14/121	+	**KX815396**	**KX815396***	**KX815398**	**KX815401**	**KX815404**	**Carpathian**
KS14/122	-	-	n.d.	n.d.	n.d.	n.d.	Carpathian
KS14/123	-	-	n.d.	n.d.	n.d.	n.d.	Eastern
KS14/124	-	-	n.d.	n.d.	n.d.	n.d.	Carpathian
KS14/125	-	-	n.d.	n.d.	n.d.	n.d.	Eastern
KS14/126	-	-	n.d.	n.d.	n.d.	n.d.	Carpathian
KS14/127	-	-	n.d.	n.d.	n.d.	n.d.	Eastern
KS14/128	+	-	n.d.	n.d.	n.d.	n.d.	**Eastern**
KS14/129	-	-	n.d.	n.d.	n.d.	n.d.	Eastern

+, positive; -, negative; P, position; n.d., not done. The nucleotide positions (P) of the novel sequences for small (S), medium (M) and large (L) segments were determined in comparison to Puumala virus strain Sotkamo (S: NC_005224; M: NC_005223; L: NC_005225). *this sequence is missing positions 43-56

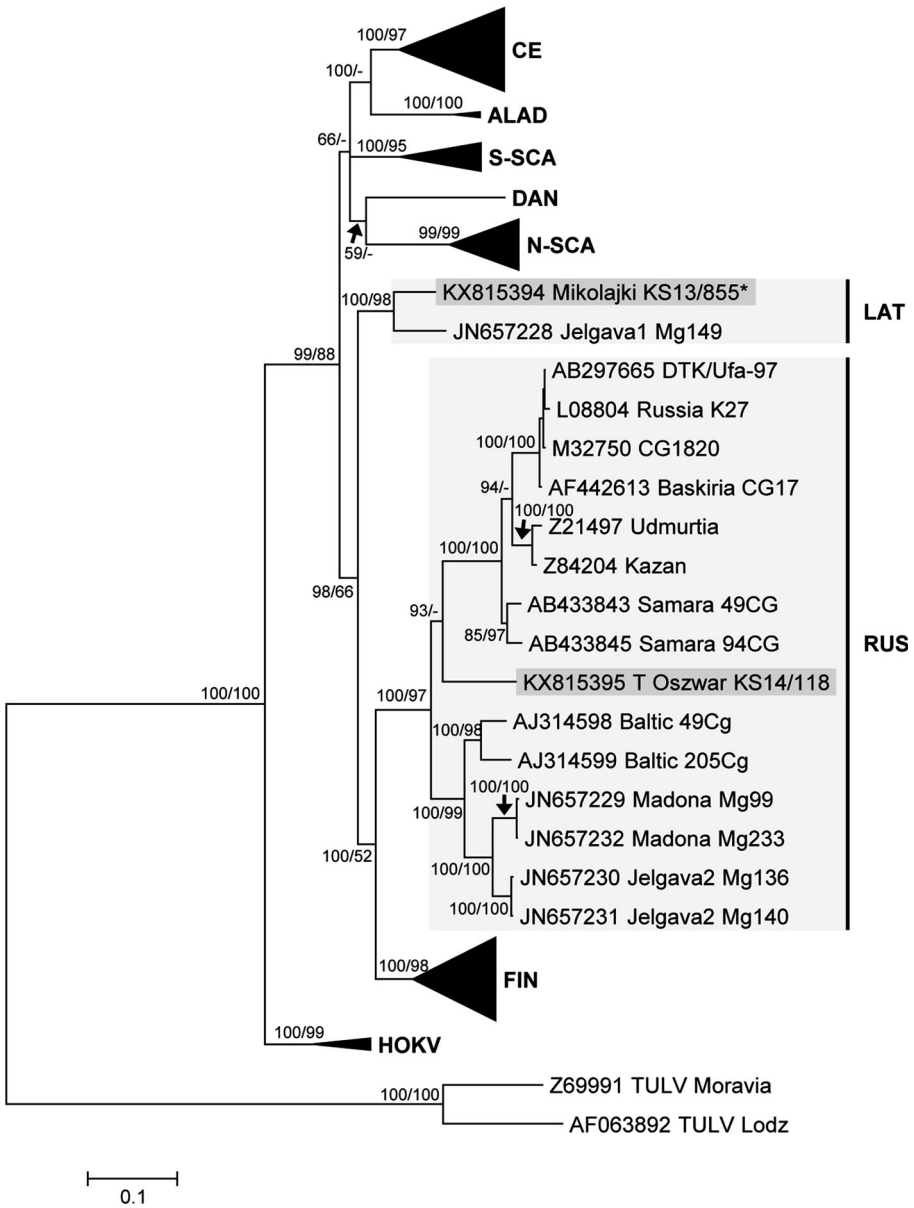

Fig. 2 Phylogenetic relationships of Puumala virus (PUUV) sequences based on comparison of the complete CDS of S segment, with Tula virus (TULV) strains Łódź AF063892 and Moravia Z69991 set as outgroups. The novel S segment sequence from Teleśnica Oszwarowa (T Oszwar; KS14/118) and the sequence from Mikołajki (KS13/855) are highlighted. Posterior probabilities are given before and bootstrap values behind slashes. PUUV lineages: ALAD Alpe-Adrian, CE Central European, DAN Danish, FIN Finnish, LAT Latvian, N-SCA North-Scandinavian, RUS Russian, S-SCA South-Scandinavian; HOKV Hokkaido virus. *Identical sequences: KS13/855 = KS13/856

analyses of the two samples from northern Poland (KS13/855, KS13/856) indicated an identical CDS (Table S2). In phylogenetic and pairwise nucleotide sequence identity analyses, the sequence from southern Poland clustered and showed the highest similarity with sequences of the PUUV lineage RUS, whereas the sequence from northern Poland clustered with a sequence of the Latvian (LAT) lineage (Mg 149 08, from Jelgava 1; Fig. 2 and Table S3).

Next, we determined a partial sequence of the M segment from both PUUV S segment RT-PCR-positive samples (Table 2). The sequence identity between both sequences from southern Poland was 98.5% at the nucleotide level and 99.5% at the amino acid level (Table S4). In contrast, both sequences varied strongly from the sequence of PUUV from northern Poland (identities of ca. 80% and 92%, respectively) and sequences from the other PUUV clades (Table S4). The phylogenetic analysis of the M segment sequences again confirmed a high divergence between the northern and southern Poland sequences as well with the other European PUUV clades (Fig. 3).

Furthermore, we tried to amplify two regions of the L segment (following previously described protocols [22, 23]) from the two samples from southern Poland as well as the three RT-PCR-positive samples from northern Poland. For one RT-PCR (positions 490-1036) four of five samples revealed specific RT-PCR products, while the second RT-PCR (positions 2956-3366) failed for two samples (Table 2 and Table S2). The sequence similarity between the novel strains from southern Poland, the

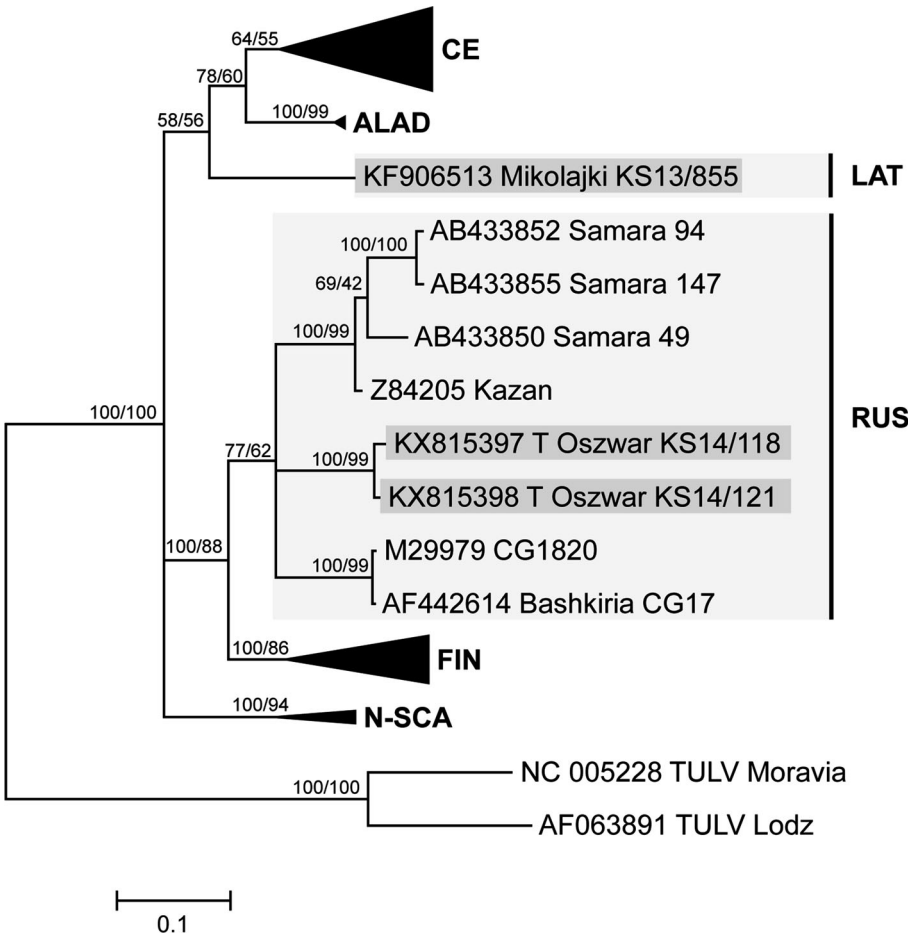

Fig. 3 Phylogenetic relationships of Puumala virus (PUUV) sequences based on comparison of 618 nucleotides of the M segment, with Tula virus (TULV) strains Łódź AF063892 and Moravia Z69991 set as outgroups. The novel M segment sequences from Teleśnica Oszwarowa (T Oszwar; KS14/118 and KS14/121) and the sequence from Mikołajki (KS13/855) are highlighted. Posterior probabilities are given before and bootstrap values behind slashes. PUUV lineages: ALAD Alpe-Adrian, CE Central European, FIN Finnish, LAT Latvian, N-SCA North-Scandinavian, RUS Russian

northern Poland strain and strains of other PUUV clades ranged from 80-88% and 92-100% at the nucleotide and amino acid level, respectively (Tables S5 and S6). Phylogenetic analysis of both regions indicated a close relationship between the sequences from southern Poland, but divergence in the sequences from northern Poland (data not shown).

Partial *cyt b* sequences were determined for all 12 voles from Teleśnica Oszwarowa as well as two voles from each of the other five trapping sites. These were compared to *cyt b* sequences of the voles from northern Poland and reference sequences for the Eastern, Western, Carpathian and Balkan lineages previously described [27–29]. At Teleśnica Oszwarowa bank voles were found to belong to the Carpathian and Eastern lineages (Table 2, Fig. 4). At the other five sites in southern Poland, voles of the Carpathian and Eastern lineages were also identified (Table 1). The two RT-PCR positive voles belonged to the Carpathian lineage, whereas the exclusively anti-PUUV antibody-positive vole belonged to the Eastern lineage (Fig. 4; Table 2). These results are in line with the investigations in northern Poland where PUUV-positive voles belonged to the Eastern and Carpathian lineages ([18]; see Table S2).

Discussion

Here we describe a PUUV survey in bank voles from different sites in southern Poland including the endemic region in the Subcarpathian area that has previously been identified [13, 14]. Only two bank voles were found to be positive in the RT-PCR assay, both coming from a single site (Teleśnica Oszwarowa) in the southeastern most part of Poland. At the neighbouring site, Solina Lake, none of the animals were found to be infected, even though the population was artificially created (based on 53 individuals trapped in Teleśnica Oszwarowa 4 years earlier) [30]. These results suggest a heterogeneous distribution of PUUV in bank voles, as previously reported for northern Poland as well as different parts of Germany [18, 31, 32].

Pairwise sequence comparisons and phylogenetic investigations of the PUUV sequences from bank voles from southeastern and northern Poland indicated separate lineages, clearly divergent from representative sequences of clades ALAD, CE, DAN, FIN, N-SCA and S-SCA; this includes sequences from the neighbouring countries Slovakia (clade CE), Germany (clade CE) and Austria (clade

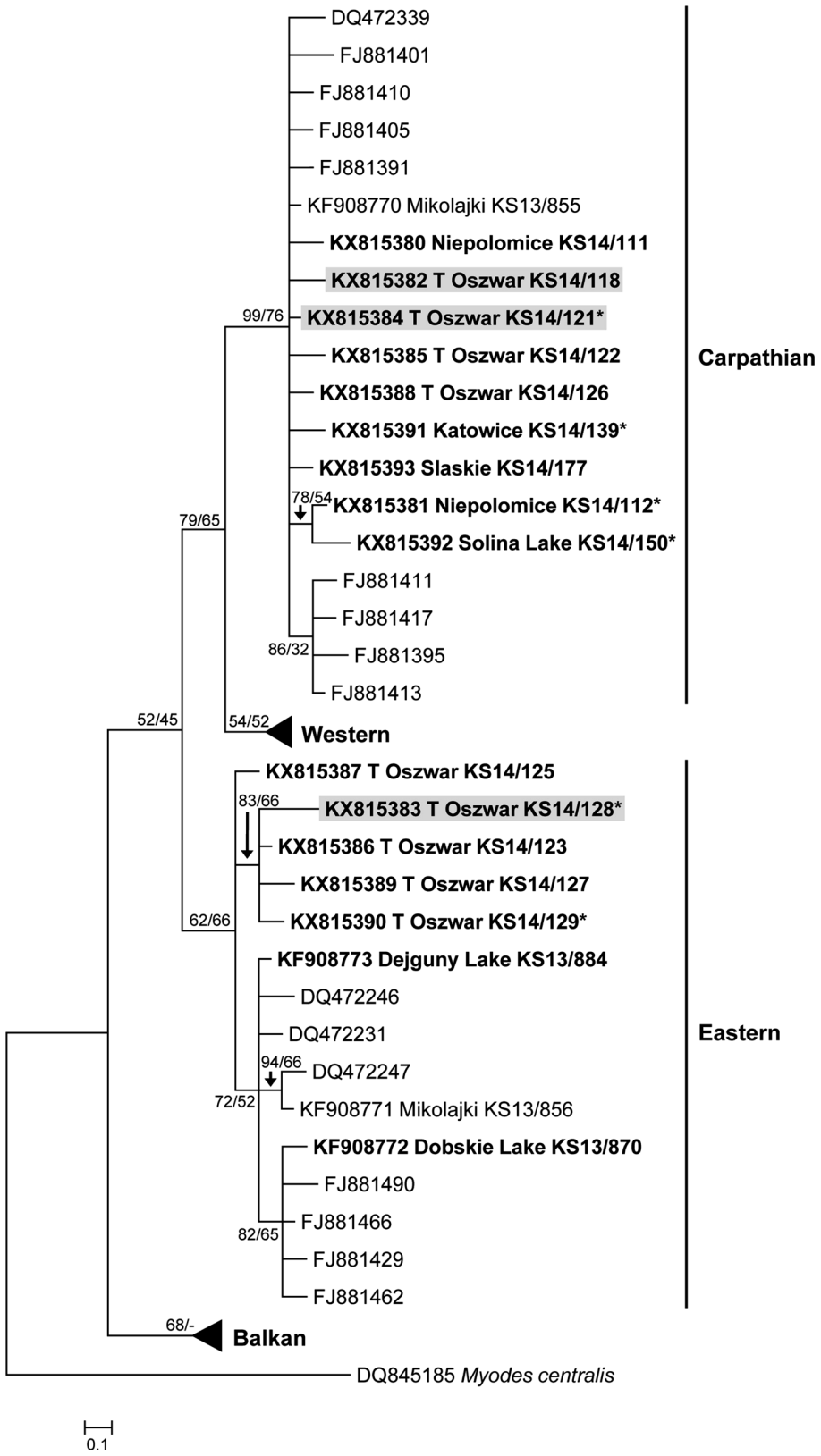

Fig. 4 Phylogenetic relationships of cytochrome b sequences in bank voles compared with major evolutionary lineages in the region. The cytochrome b sequences from *Myodes glareolus* from this study are given in bold. PUUV positive bank voles are highlighted. Posterior probabilities are given before and bootstrap values behind slashes. *Identical sequences: KS14/121 = KS14/120 = KS14/124; KS14/139 = KS14/140; KS14/112 = KS14/165 = KS14/166; KS14/150 = KS14/176; KS14/128 = KS14/119; KS14/129 = KS14/149

ALAD). The PUUV sequence from northern Poland was most similar to a LAT clade sequence from western Latvia (Jelgava 1, Fig. 1), whereas the sequences detected in southern Poland showed a different pattern with the most related sequences being from the RUS clade, found in central and western Latvia as well as Russia [33].

In agreement with previous investigations [18, 29] *cyt b* analyses revealed bank voles of the Carpathian and Eastern evolutionary lineages at the sites in southern Poland. Both RT-PCR positive animals of the RUS PUUV lineage were voles of the Carpathian lineage. This result is in line with previous studies demonstrating infections of the Carpathian bank vole lineage by the LAT and RUS PUUV lineages [18, 33]. The detection of PUUV-reactive antibodies in an additional vole of the Eastern lineage is in line with the previous studies in northern Poland and Lithuania where PUUV infections were detected in both lineages, namely Eastern and Carpathian [18, 34]. In Germany PUUV strains of the CE lineage were mainly found in the Western lineage of the bank vole, but only rarely in bank voles of the Eastern or Carpathian vole lineage where these occur sympatrically with the Western vole lineage [32]. Similarly, some TULV clades were associated with specific evolutionary lineages of the common vole (*Microtus arvalis*), with few infections of other TULV clades in the region of contact of vole lineages [35]. It was suggested that Sin Nombre virus (SNV) and related viruses are associated with certain *Peromyscus* species, or even with mitochondrial DNA haplotypes of these species [36]. In North America two geographically isolated SNV genotypes were identified in two different deer mouse (*Peromyscus maniculatus)* lineages [37]. In addition, two SNV-like Blue river virus clades were detected in two lineages of the white-footed mouse (*Peromyscus leucopus*) [38]. While these studies indicate an association of specific virus clades with host species or lineages, the generality of this observation is unclear [39], and it requires typically targeted analyses to distinguish between purely spatial and co-evolutionary associations [8, 32, 35].

Future investigations will have to study bank voles along transects in different parts of Poland and the neighbouring countries to better understand the phylogeography of the bank voles and PUUV and to develop risk assessments for humans. To clarify a possible association between specific virus and host lineages, the number of analysed animals needs to be increased in future investigations. In addition, further studies will have to evaluate potential differences in the susceptibility of the Carpathian and Eastern lineages for infection with different PUUV clades.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

Statement on the welfare of animals All procedures on animals were approved by the First Local Bioethical Committee in Kraków (decision # 48/2007).

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