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Research Paper

Identification of a novel hantavirus strain in the root vole (*Microtus oeconomus*) in Lithuania, Eastern Europe

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ABSTRACT

Hantaviruses are zoonotic pathogens that can cause subclinical to lethal infections in humans. In Europe, five orthohantaviruses are present in rodents: *Myodes*-associated Puumala orthohantavirus (PUUV), *Microtus*-associated Tula orthohantavirus, Traemmersee hantavirus (TRAV)/ Tatenale hantavirus (TATV)/ Kielder hantavirus, rat-borne Seoul orthohantavirus, and *Apodemus*-associated Dobrava-Belgrade orthohantavirus (DOBV). Human PUUV and DOBV infections were detected previously in Lithuania, but the presence of *Microtus*-associated hantaviruses is not known. For this study we screened 234 *Microtus* voles, including root voles (*Microtus oeconomus*), field voles (*Microtus agrestis*) and common voles (*Microtus arvalis*) from Lithuania for hantavirus infections. This initial screening was based on reverse transcription-polymerase chain reaction (RT-PCR) targeting the S segment and serological analysis. A novel hantavirus was detected in eight of 79 root voles tentatively named "Rusne virus" according to the capture location and complete genome sequences were determined. In the coding regions of all three genome segments, Rusne virus showed high sequence similarity to TRAV and TATV and clustered with Kielder hantavirus in phylogenetic analyses of partial S and L segment sequences. Pairwise evolutionary distance analysis confirmed Rusne virus as a strain of the species TRAV/TATV. Moreover, we synthesized the entire nucleocapsid (N) protein of Rusne virus in *Saccharomyces cerevisiae*. We observed cross-reactivity of antibodies raised against other hantaviruses, including PUUV, with this new N protein. ELISA investigation of all 234 voles detected Rusne virus-reactive antibodies exclusively in four of 79 root voles, all being also RNA positive, but not in any other vole species. In conclusion, the detection of Rusne virus RNA in multiple root voles at the same trapping site during three years and its absence in sympatric field voles suggests root voles as the reservoir host of this novel virus. Future investigations should evaluate host association of TRAV, TATV, Kielder virus and the novel Rusne virus and their evolutionary relationships.

1. Introduction

The genus *Orthohantavirus* belongs to the family *Hantaviridae* within the order *Bunyavirales* and currently contains 36 virus species (Schmaljohn and Dalrymple, 1983; ICTV, 2020). These segmented, negative strand RNA viruses are believed to have coevolved with their respective hosts and are strongly associated with one species or in some cases, such as Tula orthohantavirus (TULV), with several related species (Schmidt-Chanasit et al., 2010; Guterres et al., 2015; Milholland et al., 2018). However, cross-species transmission (host switch) is another

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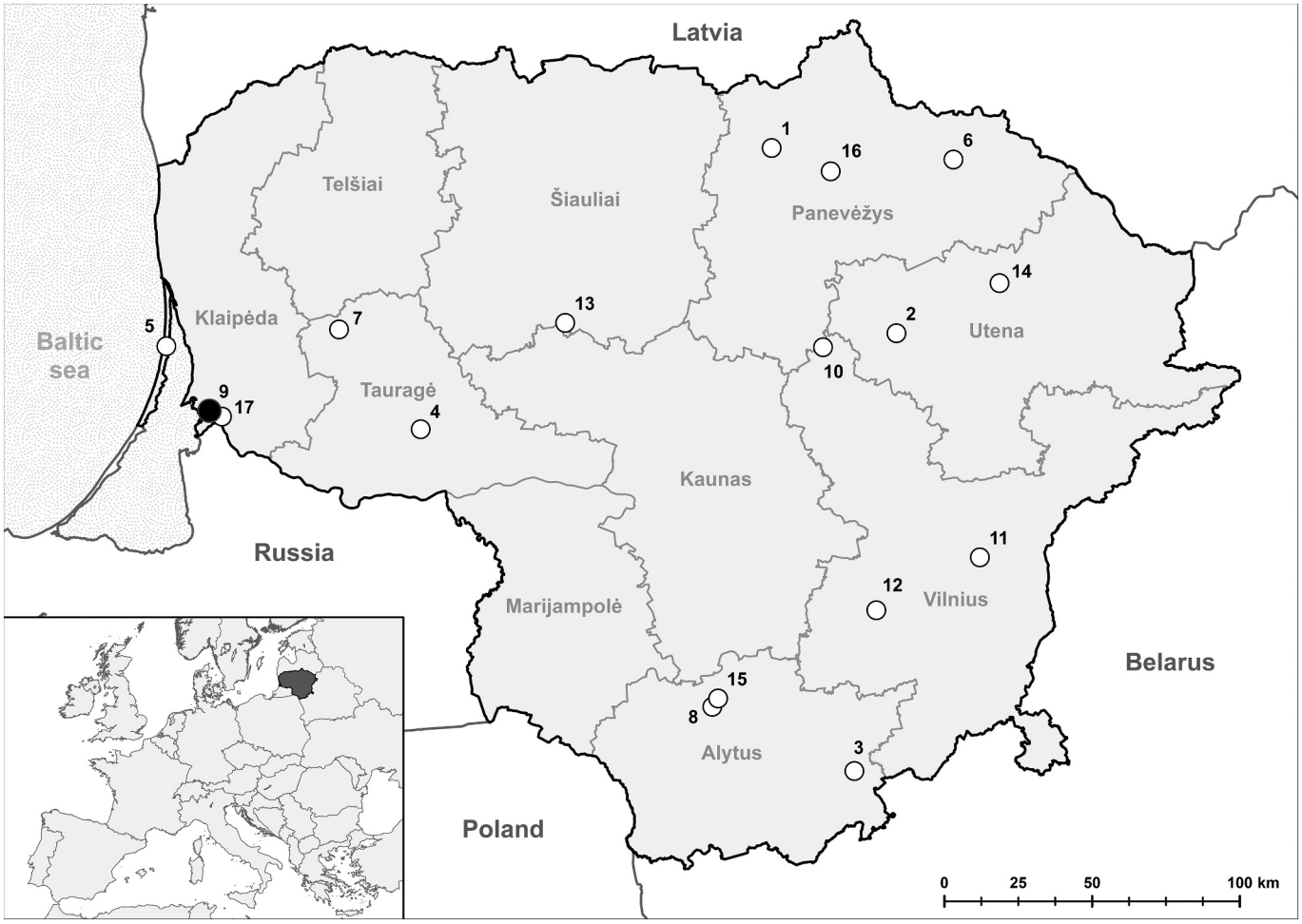

Fig. 1.. Trapping sites of *Microtus* voles in Lithuania, Eastern Europe. Numbers correspond to trapping areas (see Table 1). Negative tested trapping sites are marked with white circles, and site Rusnė (9) with detection of Rusne virus is marked with a black circle.

important factor in hantavirus evolution (Ramsden et al., 2009; Guo et al., 2013; Bennett et al., 2014). Transmission to humans is usually mediated by inhalation of virus-contaminated aerosols such as feces and urine of infected hosts. Infections in humans can result in a subclinical course to severe illness, including hemorrhagic fever with renal syndrome (HFRS) with case fatality rate reaching 12% or hantavirus cardiopulmonary syndrome (HCPS) with case fatality rate up to 40% (Avšič-Županc et al., 2019).

The three genome segments are flanked by non-coding regions (NCR) that form panhandle-like structures (Spiropoulou, 2011). The S segment of 1530 to 2078 nucleotides (nt) encodes the nucleocapsid (N) protein of 428 to 433 amino acid (aa) residues (Plyusnin et al., 1994a). A non-structural protein (NSs) is encoded in an overlapping open reading frame (ORF) in the S segment of orthohantaviruses carried by rodents of the family Cricetidae and might be important as an interferon inhibitor (Jääskelainen et al., 2007). The M segment is 3543–3801 nt long and encodes the glycoprotein precursor (GPC) that is cotranslationally cleaved into the amino-terminal Gn protein of 503–528 aa residues and the carboxy-terminal Gc protein of 479–486 aa residues (Sironen and Plyusnin, 2011). The L segment of 6529–6578 nt has the coding information for a 2147–2155 aa-residue long RNA-dependent RNA polymerase (RdRP) (Schmaljohn, 1990; Schlegel et al., 2014).

Voles of different *Myodes* and *Microtus* species represent hantavirus reservoirs. Whereas no hantavirus was detected in *Myodes* spp. in the New World so far (Milholland et al., 2018), several *Microtus* associated hantaviruses were found there. The reservoir of Prospect Hill orthohantavirus (PHV) has been identified as the meadow vole (*Microtus pennsylvanicus*) (Lee et al., 1985), but the virus has been also detected in montane vole (*Microtus montanus*) and prairie vole (*Microtus ochrogaster*) (Rowe et al., 1995). The montane vole was identified as the reservoir of El Moro Canyon orthohantavirus (Rowe et al., 1995). The California vole (*Microtus californicus*) represents the reservoir of Isla Vista virus, but was also found to be affected by spillover infection with Sin Nombre orthohantavirus, with the deer mouse (*Peromyscus maniculatus*) being the reservoir (Song et al., 1995; Turell et al., 1995).

In the Old World, *Myodes* spp. such as bank vole (*Myodes glareolus*), royal vole (also called Korean red-backed vole, *Myodes regulus*) and grey red-backed vole (*Myodes rufocanus*) transmit Puumala orthohantavirus (PUUV), which causes the majority of human hantavirus disease cases (Kariwa et al., 1995; Lundkvist et al., 1998; Song et al., 2007). TULV is one of the best studied and most broadly distributed orthohantaviruses. It is associated with the common vole (*Microtus arvalis*) and is genetically highly divergent with more than six phylogenetic clades, including the Eastern South (EST.S) clade with the Moravia prototype strain in the Czech Republic and the Central North (CEN.N) clade in the Northern part of Germany (Schmidt et al., 2016; Saxenhofer et al., 2019). TULV has been detected also in field voles (*Microtus agrestis*), narrow-headed voles (*Microtus gregalis*), East-European voles (*Microtus levis*), Altai voles (*Microtus obscurus*), European pine voles (*Microtus subterraneus*), and water voles (*Arvicola amphibius*) (Plyusnin et al., 1994b; Song et al., 2002; Scharninghausen et al., 2002; Plyusnina et al., 2008; Schmidt-Chanasit et al., 2010; Schlegel et al., 2012a; Polat et al., 2018). The field vole was recently identified as a reservoir of three

closely-related hantaviruses: Traemmersee hantavirus (TRAV) in Germany (Jeske et al., 2019), Tatenale hantavirus (TATV) and Kielder hantavirus in Great Britain (Pounder et al., 2013; Thomason et al., 2017; Chappell et al., 2020). Additional hantaviruses, namely Khabarovsk orthohantavirus (KHAV), Fusong orthohantavirus, and Yuanjiang virus were found in Asia and are associated with Maximowicz's vole (*Microtus maximowiczii*) (Zou et al., 2008), reed vole (*Microtus fortis*) (Kariwa et al., 1995; Hörling et al., 1996; Zou et al., 2008) and root or tundra vole (*Microtus oeconomus*) (Plyusnina et al., 2008).

From the Baltic states in Europe, including Lithuania, Latvia and Estonia, only Dobrava-Belgrade orthohantavirus (DOBV) and PUUV have been reported so far. Human infections with these viruses have been detected by serological methods (Lundkvist et al., 2002; Sandmann et al., 2005; Golovljova et al., 2007). Molecular evidence for the presence of these hantaviruses originated from the screening of bank voles in Lithuania and striped field mice (*Apodemus agrarius*) on the Estonian island Saaremaa (Nemirov et al., 1999; Straková et al., 2017). To date, nothing is known about the presence of hantaviruses in *Microtus* voles in this part of Europe. In this study, common voles, field voles, and root voles from multiple sites in Lithuania were screened by reverse transcription-polymerase chain reaction (RT-PCR) and immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) for the presence of TULV and related hantaviruses.

2. Material and methods

2.1. Collection of voles and dissection

Voles were trapped at 17 sites in Lithuania (Fig. 1; Table 1) during September, October and beginning of November 2015 to 2018, following methods described before (Balčiauskas, 2004). Morphological species identification was based on a dichotomous key previously described (Prūsaitė et al., 1988; Pucek, 1984). For selected animals, species identification was confirmed by dideoxy chain termination sequencing of cytochrome *b* PCR products and sequence comparison to GenBank entries as described elsewhere (Schlegel et al., 2012b). Weight, gender, and age class were determined for each carcass. Several tissue samples (lung, liver and kidney) were collected and stored at −20 °C. For detection of hantavirus-reactive antibodies tissue fluids were collected by thawing of previously frozen liver and lung tissue.

2.2. RNA isolation, RT-PCR, RNA ligation, conventional and high-throughput sequencing

RNA was extracted from lung tissue to screen for hantavirus infection using S segment specific RT-PCR as previously described (Schmidt et al., 2016). To determine complete genome sequences, total RNA of samples LT15/299, LT15/301, LT15/341 and LT15/351 was analyzed using hybrid sequence capture enrichment with subsequent high-throughput sequencing following Hiltbrunner and Heckel (2020). Consensus sequences of virus genomes were determined based on de novo assemblies of sequence reads called at a minimum base quality of $Q_{phred} = 33$ and 90% identity. Additional reference-based mapping of not assembled sequence reads was used to close short regions with low read coverage in some samples (see results; Saxenhofer et al., 2019; Hiltbrunner and Heckel, 2020). Furthermore, the complete coding sequences (CDS) of sample LT15/301 were determined by primer-walking (for primers see Table S1). Additionally, partial S, M and L segment sequences were generated for several RT-PCR positive samples by dideoxy chain termination sequencing of RT-PCR products including primer walking for selected samples (see Table S2). Sequences of the 5′ and 3′ NCRs of strain LT15/301 were determined by RT-PCR analysis of RNA molecules generated by RNA ligation using T4 RNA Ligase (Thermo Fisher, Waltham, MA, USA) following a published protocol (Klempa et al., 2006).

Table 1
Results of RT-PCR and IgG ELISA hantavirus screening of voles collected in Lithuania during 2015–2018.

Site (number in Fig. 1)	Year	Species	Results (number of positive/total number of investigated voles)	
			RT-PCR	IgG ELISA
Aukštikalniai (1)	2018	*Microtus arvalis*	0/1	0/1
Ažuožeriai (2)	2018	*Microtus arvalis*	0/30	0/30
		Microtus oeconomus	0/4	0/4
Barčiai (3)	2018	*Microtus arvalis*	0/11	0/11
Gaurė (4)	2018	*Microtus arvalis*	0/4	0/4
		Microtus oeconomus	0/2	0/2
Juodkrantė (5)	2015	*Microtus agrestis*	0/1	0/1
		Microtus oeconomus	0/2	0/2
Kalpokai (6)	2018	*Microtus arvalis*	0/7	0/7
Kvėdarna (7)	2018	*Microtus agrestis*	0/1	0/1
Luksnėnai (8)	2018	*Microtus arvalis*	0/6	0/6
		Microtus oeconomus	0/3	0/3
Rusnė (9)	2015	*Microtus agrestis*	0/12	0/12
		Microtus oeconomus	**6**/42	**4**/42
	2016	*Microtus agrestis*	0/7	0/7
		Microtus oeconomus	**1**/7	0/7
	2017	*Microtus agrestis*	0/4	0/4
		Microtus arvalis	0/1	0/1
		Microtus oeconomus	**1**/8	0/8
	2018	*Microtus agrestis*	0/10	0/10
		Microtus oeconomus	0/3	0/3
Taujėnai (10)	2018	*Microtus arvalis*	0/9	0/9
		Microtus oeconomus	0/1	0/1
Tauragirė (11)	2017	*Microtus oeconomus*	0/1	0/1
Trakai (12)	2016	*Microtus arvalis*	0/11	0/11
Tytuvėnai (13)	2018	*Microtus agrestis*	0/1	0/1
		Microtus arvalis	0/1	0/1
		Microtus oeconomus	0/1	0/1
Užpaliai (14)	2018	*Microtus arvalis*	0/9	0/9
Užubaliai (15)	2018	*Microtus arvalis*	0/4	0/4
Vabalninkas (16)	2017	*Microtus arvalis*	0/15	0/15
		Microtus oeconomus	0/1	0/1
Žalgiriai (17)	2017	*Microtus agrestis*	0/10	0/10
		Microtus oeconomus	0/4	0/4
Total		*Microtus agrestis*	0/46	0/46
		Microtus arvalis	0/109	0/109
		Microtus oeconomus	**8**/79	**4**/79

Rusne hantavirus RNA and/or anti-Rusne hantavirus antibody positive root voles are given in bold.

2.3. Phylogenetic analyses

Sequences were aligned using the Clustal W algorithm in Bioedit version 7.2.5. (Hall, 1999) (see Table S3). For nt sequences, the best-fitting substitution model was determined with jModelTest2 version 2.1.6, whereas on aa sequences ModelTest-NG version 0.1.5 was applied (Darriba et al., 2012; Darriba et al., 2020). The General Time Reversible (GTR) substitution model with invariant sites and a gamma distributed shape parameter was used in phylogenetic analyses with MrBayes version 3.2.7a with 1 to 3.5 × 10^7 generations and 25% burn-in and Maximum-Likelihood analyses with FastTreeMP version 2.1.10 with 1000 bootstraps. At the aa level, analyses with MrBayes used 8 × 10^6 generations, the retrovirus-specific (rtREV) and Whelan

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Goldman (WAG) substitution models and a burn-in phase of 25%. Maximum-Likelihood analyses were performed with FastTreeMP using the Jones-Taylor-Thornton (JTT) and the categories (CAT) model and 1000 bootstrap replicates. Consensus phylogenetic trees were drawn with 50% cut-off and posterior probability values greater than 95% and bootstrap values greater than 75 were reported at the nodes. All phylogenetic reconstructions were performed on CIPRES (Miller et al., 2010).

2.4. Pairwise evolutionary distance analysis

To test if the new Rusne virus and TRAV as well as TATV belong to the same virus species, pairwise evolutionary distance (PED) values were determined (Laenen et al., 2019). Available entire S and M segment CDS of hantaviruses were concatenated and translated to amino acid sequences. PED values were calculated using a maximum-likelihood approach with the WAG substitution model in Tree-Puzzle. Thottapalayam thottimvirus was used as an outgroup. A PED cut-off value of 0.1 was used for species demarcation within the family *Hantaviridae*.

2.5. Generation of recombinant N proteins and serological analysis

The complete N protein encoding sequence of strain LT17/R6 was amplified using primers RuNRES_FOR and RuNRES_REV (see Table S1) and inserted into the *Xba*I-linearized pFX7-His plasmid (Ražanskienė et al., 2004). *Saccharomyces cerevisiae* strain Gcn2 was transformed with this plasmid, the synthesis of recombinant N protein of Rusne virus was initiated by adding galactose solution into YEPD medium. Recombinant N protein was purified by nickel chelate affinity chromatography as described before (Ražanskienė et al., 2004). The generation and purification of N proteins of TULV clade CEN·N, strain Thuringia, TULV clade EST.S, strain Moravia, and PUUV strains Vranica/Hällnäs and Bavaria have been described previously (Ražanskienė et al., 2004; Mertens et al., 2011; Jeske et al., 2019). Characterization of the Rusne virus antigen was done by ELISA and Western blot analysis using monoclonal antibodies (mAbs) produced against recombinant N proteins of PUUV, strain Vranica/Hällnäs and Sin Nombre/Andes orthohantaviruses (Kučinskaitė-Kodzė et al., 2011; Zvirbliene et al., 2006).

ELISA investigations of vole-derived chest cavity lavage (CCL) and tissue fluid samples with recombinant N proteins of Rusne virus, TULV strain Thuringia and PUUV strain Bavaria followed a standard protocol established for TULV (Schlegel et al., 2012a). Briefly, 0.2 µg/well of the recombinant protein were coated on 96-well polysorb Nunc-Immuno plates (VWR International GmbH, Hannover, Germany) and incubated for 1 h with tissue fluid or CCL diluted 1:10. After washing, a goat anti-mouse IgG (H + L) labeled with horse-radish peroxidase (BioRad, Munich, Germany) was used to detect antibodies against the specific hantavirus antigen. Finally, 100 µl of tetramethylbenzidine (TMB) peroxidase EIA substrate (BioRad, Munich, Germany) was added and incubated for 10 min in the dark. The reaction was stopped by addition of 100 µl 1 M sulfuric acid. Subsequently, optical density was measured with Plate reader Infinite 200 PRO (Tecan, Männedorf, Switzerland) at 450 nm (reference at 620 nm). The mAb 5E11 (Kučinskaitė-Kodzė et al., 2011) was used as positive control. Bank vole (PUUV) and common vole (TULV) CCL samples from previous studies (Drewes et al., 2017; Jeske et al., submitted) or root vole tissue fluid tested negative by the respective hantavirus-IgG ELISA and RT-PCR were applied as negative controls. Lower and upper cut-off values were determined according to a previous study (Mertens et al., 2011). For the cross-reactivity study three anti-PUUV-positive bank vole and three anti-TULV-positive common vole CCL samples were used originating from previous investigations (Drewes et al., 2017; Jeske et al., submitted).

3. Results

3.1. RT-PCR screening of voles

A total of 234 voles including 79 root voles, 46 field voles and 109 common voles were collected during small mammal trapping sessions at 17 sites in Lithuania during 2015–2018 (Fig. 1). Hantavirus RT-PCR screening resulted in the identification of eight positive samples (Table 1). All positive samples were root voles collected in 2015, 2016 and 2017 at Rusnė site. The eight hantavirus RNA positive samples originated from five adult and one juvenile male, and one adult and one subadult female (Table S2). Sympatrically occurring field voles (N = 33) and a single common vole from this site were hantavirus RNA negative. None of the voles from any of the other trapping sites were found to be hantavirus RNA positive.

RT-PCR-mediated generation of partial S and L segment sequences indicated a novel hantavirus strain in the root voles, designated according to the trapping site “Rusne virus”. The strain LT15/301 has the highest nucleotide and amino acid sequence similarity to the other Rusne virus strains (0.987–1.0/1.0) and then to the recently discovered Traemmersee hantavirus (TRAV; 0.809–0.837/0.963–0.977) and Tatenale hantavirus (TATV; 0.796–0.834/0.954–0.99) (see Fig. S2, Table S4). The L segment/RdRP sequences of British Kielder hantavirus strains showed similarities of 0.816–0.819/0.981–0.99 to the Rusne virus strains (Fig. S2, Table S4).

3.2. Complete genome sequence determination and phylogenetic analysis

To generate a complete genome sequence of this novel hantavirus, a hybrid sequence capture approach was followed for samples LT15/299, LT15/301, LT15/341 and LT15/351. This allowed us to obtain genome sequences of the Rusne virus with mean sequence read depths of the assemblies of 422x (LT15/299), 51x (LT15/301), 30x (LT15/341) and 61x (LT15/351). For three samples, the gapless assemblies covered the complete coding sequences (CDS) of S segment, M segment and L segment, and at least parts of the 5′ and 3′ NCRs (Tables S2 and S6). The NGS-derived L segment sequence of LT15/341 lacked 199 nt and 4% of the nt were called at a read depth less than 5x compared to 0%, 0.3% and 0.1% in the other assemblies. However, the determined parts of L segment and the entire CDS of S and M segments were identical to those of LT15/351. In parallel, a primer walking based approach resulted in the generation of complete CDS of S, M and L segments of strain LT15/301 (Table S2). The NGS-derived and primer-walking approach-based sequences of LT15/301 were identical except for one nucleotide difference over the whole L CDS and one different nucleotide in the M CDS. Sequence read coverage of the NGS assembly was at least 30x at these positions and there were no sequence reads with the nucleotide determined in the primer-walking-based sequence.

The S segment had a total length of 2059 nt and encodes a N protein of 433 aa residues (Table S6). The putative NSs protein of Rusne virus strains LT15/301 and LT17/R6 had an amino-terminal extension of four residues, similar to the extension of five residues in the corresponding putative protein of KHAV, but different from that of TRAV and TATV and other vole-borne hantaviruses. The S segment 5′ and 3′ NCR sequences of strain LT15/301 were identified to be of 42 nt and 715 nt, respectively (Table S6). The 5′ NCR sequences were highly conserved in length before the start codon and for the first 30 nt in particular (Fig. 2), whereas the 3′ NCR sequence showed a higher sequence variability with two 26–53 nt long insertions/deletions, but a more conserved sequence at the terminal 100 nt (data not shown).

The M segment was predicted to encode a GPC of 1148 aa residues and contained the conserved WAASA cleavage motif between Gn and Gc at aa residues 654–658; the NGS allowed the determination of 28 and 214 nt of the NCR sequences (Tables S2 and S6). The L segment had a coding sequence of 6465 nt and codes for the RdRP of 2155 aa residues. Again, only partial sequences at both NCR ends were

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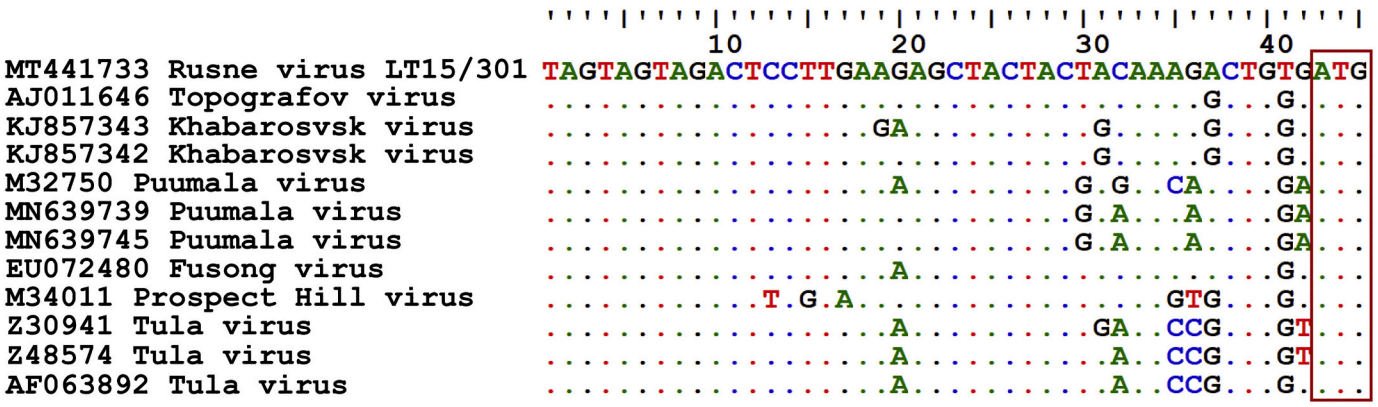

Fig. 2.. Comparison of S segment 5′ NCR sequences of the novel Rusne virus, strain LT15/301 with sequences of reference strains. Identical nucleotides displayed as dots. The start codon of the N protein of the different hantaviruses is framed in red.

determined by NGS (Tables S2 and S6).

The S, M and L segment CDS as well as the N, GPC and RdRP amino acid sequences showed the highest similarity to TRAV and TATV sequences, with overall pairwise CDS nt and aa sequence similarity ranging between 0.802 and 0.847 and 0.939–0.976, respectively (Table S5). The nt and aa sequences of the three viruses formed a monophyletic group in all phylogenetic analyses (Fig. 3A–F).

Comparison of concatenated N and GPC aa sequences of Rusne virus resulted in PED values below 0.1 to TRAV and TATV (Table S5). This indicates that the novel Rusne virus discovered in *M. oeconomus* and TRAV and TATV detected in *M. agrestis* belong to the same tentative orthohantavirus species.

3.3. Cross-reactivity of recombinant N protein of Rusne virus and serological screening of voles

The entire N protein of Rusne virus was produced in *S. cerevisiae* yeast and purified by affinity chromatography. The N protein of Rusne virus was tested in parallel with N antigens of TULV strain Moravia, PUUV strains Bavaria and Vranica/Hällnäs, and SNV for cross-reactivity. A high cross-reactivity was documented by ELISA and Western blot analyses using PUUV-specific mAbs 5C5, 5E11 and 7A5 and SNV/ANDV-specific mAb 7G2 (Table 2). In addition, three of three anti-TULV-positive common vole CCL samples and two of three anti-PUUV-positive bank vole CCL samples reacted in the ELISA with Rusne virus antigen (data not shown).

ELISA screening of tissue fluids of all 234 voles resulted in the detection of four seropositive root voles (Table 1 and Table S2). All four ELISA-positive samples originated from Rusne virus-RNA positive voles; four additional RNA-positive samples were negative in this novel ELISA (Table S2). The IgG ELISA positive samples consisted of two males and two females, from which one female was subadult and the other three voles were adult individuals.

4. Discussion

We detected a *Microtus*-associated hantavirus in Lithuania, Eastern Europe. The multiple detection of this novel virus in root voles at one site over time suggests this vole species as the reservoir of this novel hantavirus. This conclusion was further strengthened by the absence of Rusne virus RNA in the field voles collected at the same trapping site.

We conclude that the new Rusne virus is a strain of TRAV recently detected in Germany and TATV discovered in Great Britain, due to their sequence similarity with a PED value below the definition level of a new species as stated by the International Committee on Taxonomy of Viruses (ICTV) (Pounder et al., 2013; Thomason et al., 2017; Jeske et al., 2019; Chappell et al., 2020; ICTV, 2020). On the basis of partial L segment sequences Rusne virus, TRAV and TATV are also very similar to British Kielder hantavirus. It appears that they all belong to one tentative orthohantavirus species, given high levels of genetic divergence also within other European orthohantavirus species and the importance of geographic isolation for evolutionary processes in hantaviruses (Weber de Melo et al., 2015; Saxenhofer et al., 2017; Saxenhofer et al., 2019; Hiltbrunner and Heckel, 2020).

Interestingly, TRAV in Germany as well as Tatenale and Kielder hantaviruses in Great Britain were only detected in field voles (Pounder et al., 2013; Thomason et al., 2017; Jeske et al., 2019). In this study, Rusne virus was exclusively detected in root voles, but not in sympatric field voles. The root vole is the only member of the genus *Microtus* with a holarctic distribution from the German-Polish border through Asia, Alaska and Canada, including isolated populations in some regions of Europe, e.g. in the Netherlands (von Tast, 1982; Brunhoff et al., 2003; Fink et al., 2010). The root vole invaded Lithuania relatively recently, with first findings in 1964 from the south and south-western parts and subsequently colonized most of Lithuania (Ivanauskas et al., 1964; Balčiauskas et al., 2010). In the Nemunas River Delta with the Rusnė trapping site, the root vole joins the striped field mouse (*Apodemus agrarius*) as dominant small mammals, and is found in higher

Table 2
Reactivity of recombinant nucleocapsid (N) proteins of orthohantaviruses with monoclonal antibodies (mAbs) in ELISA and Western blot test.

		Reactivity of mAbs in ELISA/Western blot test					
		4H3	7G2	2C6	5C5	5E11	7A5
Recombinant N proteins	Rusne virus	−/−	+/+	−/−	+/+	+/+	+/+
	TULV-Moravia	−/−	+/+	−/−	+/+	+/+	(+)/+
	PUUV-Bawa	−/−	+/+	−/−	+/+	+/+	+/+
	PUUV-Vra	n.d./n.d.	+/n.d.	+/+	+/n.d.	+/n.d.	+/n.d.
	SNV	+	+/+	−/−	+/+	+/+	+/+

n.d., not determined; +, positive; (+), weakly positive; −, negative.
TULV-Moravia, Tula virus, strain Moravia; PUUV-Bawa, Puumala virus, strain Bavaria; PUUV-Vra, Puumala virus, strain Vranica/Hällnäs; SNV, Sin Nombre virus.

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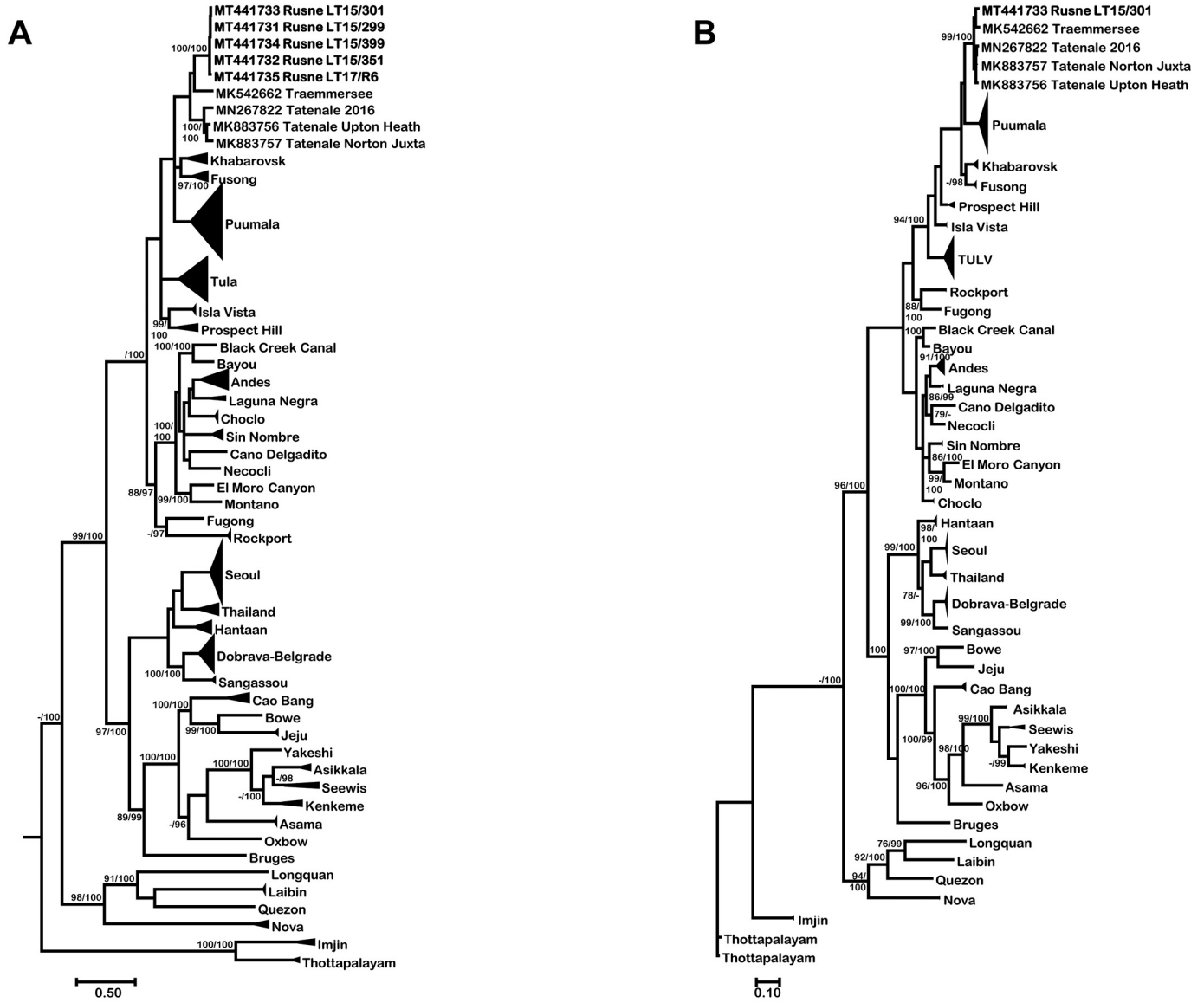

Fig. 3.. Consensus phylogenetic trees of complete coding sequences (CDS) of S segment (A), M segment (C) and L segment CDS (E) and amino acid sequences of complete nucleocapsid protein (B), glycoprotein precursor protein (D), and RNA-dependent RNA polymerase (F). Alignments were constructed using the Clustal W Multiple Alignment algorithm implemented in Bioedit V7.2.3. (Hall, 1999). The consensus nt sequence trees are based on Bayesian analyses with up to 3.5×10^7 generations and a burn-in phase of 25%, and Maximum-Likelihood analyses with 1000 bootstraps and 50% cut-off using the General Time Reversible (GTR) substitution model with invariant sites and a gamma distributed shape parameter for both algorithms. Phylogenetic consensus trees of the complete amino acid sequences of the nucleocapsid (B), complete glycoprotein precursor protein (D), and the complete RNA-dependent RNA polymerase (F) were constructed with Bayesian algorithms with 8×10^6 generations using the retrovirus-specific (rtREV) and Whelan Goldman (WAG) substitution models and with Maximum Likelihood algorithms, the Jones-Taylor-Thornton (JTT) and CAT substitution models and 1000 bootstrap replicates. Bootstrap values were only transferred to the Bayesian trees, if branches were consistent. Posterior probability values > 95%/bootstrap values > 70 are given at the supported nodes. The tree reconstructions were done on CIPRES (Miller et al., 2010). Names in bold indicate newly generated sequences (GenBank accession numbers MT441731 – MT441741). Triangles indicate condensed branches; for all hantavirus sequences included see Table S3.

proportions than in other regions of Lithuania (Balčiauskas et al., 2012). Interestingly, one root vole was previously found to harbor Fusong orthohantavirus (FUSV), strain Vladivostok, in Russia (Plyusnina et al., 2008). However, the reservoir host of FUSV is the reed vole and the detection of FUSV in the single root vole might represent a spillover infection (Kariwa et al., 1999; Plyusnina et al., 2008; Zou et al., 2008).

At present, it is unclear if the detection of Rusne virus in root voles in Lithuania, TRAV in a field vole in Germany and TATV and Kielder hantavirus in field voles in Britain might be the result of a host switch of an ancestral virus in the past. Both vole species are present in Eastern Germany, as well as Eastern Europe in general with similar habitats, but root voles are absent from the British Isles (Kryštufek et al., 2007; Linzey et al., 2016). To determine if host switch has taken place and what was the original host of Rusne virus, TRAV and TATV further studies in field voles, root voles and other *Microtus* voles in Eurasia are needed.

Rusne virus RNA was more frequently detected than Rusne virus-reactive antibodies. As we used here the new Rusne virus N protein - a homologous antigen - in the ELISA, this discrepancy cannot be explained by the use of a heterologous antigen. One explanation might be the use of highly diluted tissue fluids. Alternatively, this discrepancy might be caused by seronegative root voles being in the acute phase of the infection. Therefore, a screening of voles for hantavirus infection

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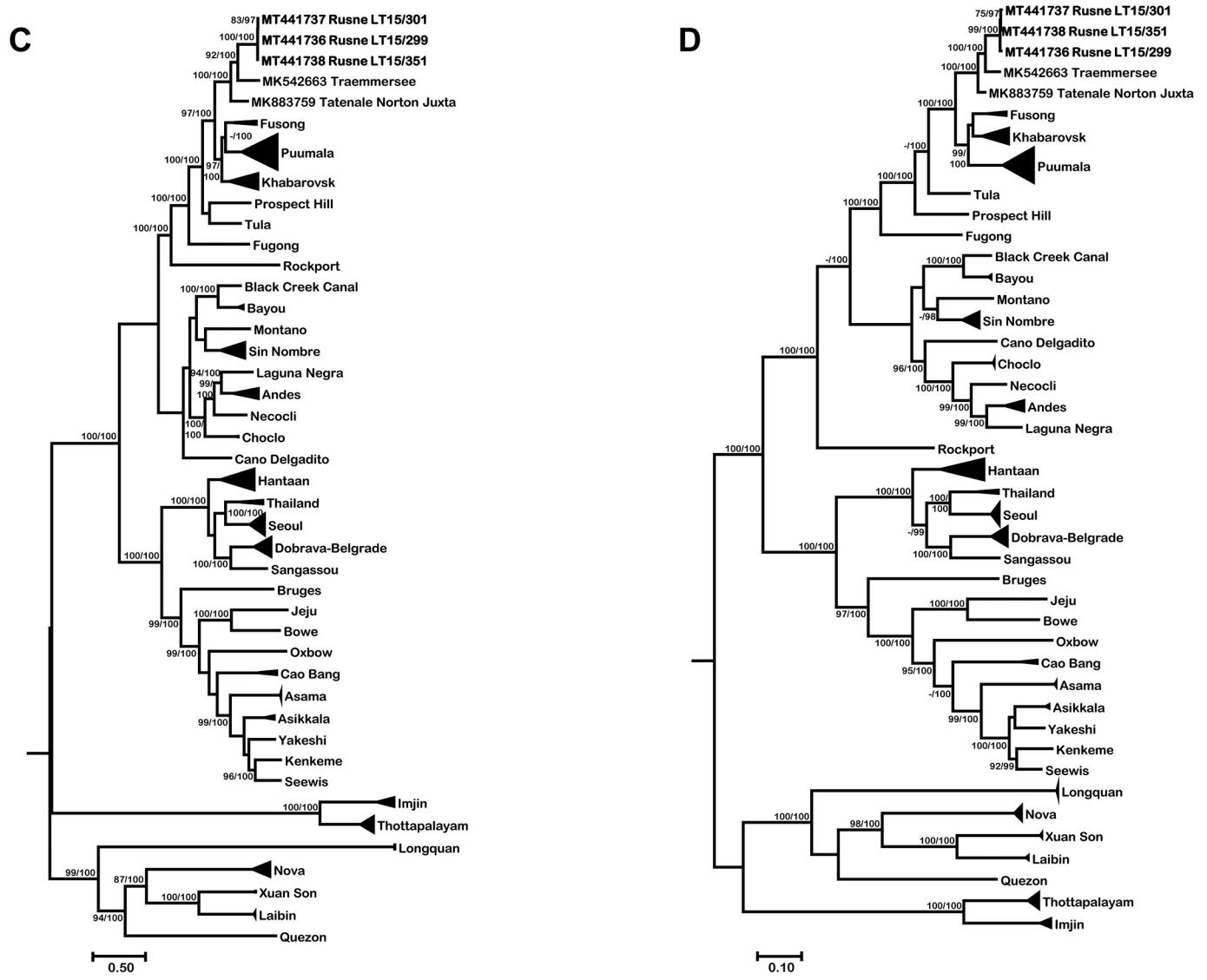

Fig. 3.. (*continued*)

may profit from a molecular approach instead of a serologic assay (Weber de Melo et al., 2015), in particular for selection of samples for virus isolation (Binder et al., 2020).

The high aa sequence similarity between N proteins of Rusne virus, PUUV and TULV was reflected in a strong cross-reactivity of these antigens as evidenced by ELISA and Western blot investigations exploiting mAbs and polyclonal sera. Therefore, the detection of PUUV- or TULV-reactive antibodies in human serum samples, as e.g. in Lithuania (Sandmann et al., 2005; Dargevičius et al., 2007), might be misinterpreted in regions where TRAV/Rusne virus/TATV/Kielder virus circulates. For a definite proof of potential human infections with this virus species, a virus isolate for focus reduction neutralization test use is urgently needed (Krüger et al., 2001).

5. Conclusions

In this study we detected the first root vole-associated hantavirus in Europe, Rusne virus, that forms a putative hantavirus species together with TRAV and TATV. The multiple detection of similar sequences of this novel virus in a root vole population during three years suggests this vole species as the reservoir host. We further developed a Rusne virus antigen that might be used in serological screenings of human serum samples. A Eurasian wide screening of root voles, field voles and other *Microtus* voles is needed to evaluate the geographic range and possible host association of Rusne virus, TRAV and TATV strains. In the future, isolation of strains of these viruses is needed for the development of additional serological detection tests of human infections including neutralization assays.

Author contributions

Stephan Drewes: Conceptualization, Visualization, Writing - original draft, Investigation, Writing - review & editing. Kathrin Jeske: Conceptualization, Visualization, Writing - original draft, Investigation, Writing - review & editing. Petra Straková: Investigation, Writing - review & editing. Linas Balčiauskas: Investigation, Data curation, Writing - review & editing. René Ryll: Investigation, Formal analysis, Writing - review & editing. Laima Balčiauskienė: Investigation, Data curation, Writing - review & editing. David Kohlhause: Investigation. Guy-Alain Schnidrig: Investigation, Formal analysis. Melanie Hiltbrunner: Investigation, Data curation. Aliona Špakova: Investigation, Data curation. Rasa Insodaitė: Investigation, Formal analysis. Rasa Petraitytė-Burneikienė: Supervision, Writing - review & editing. Gerald Heckel: Conceptualization, Writing - original draft, Supervision,

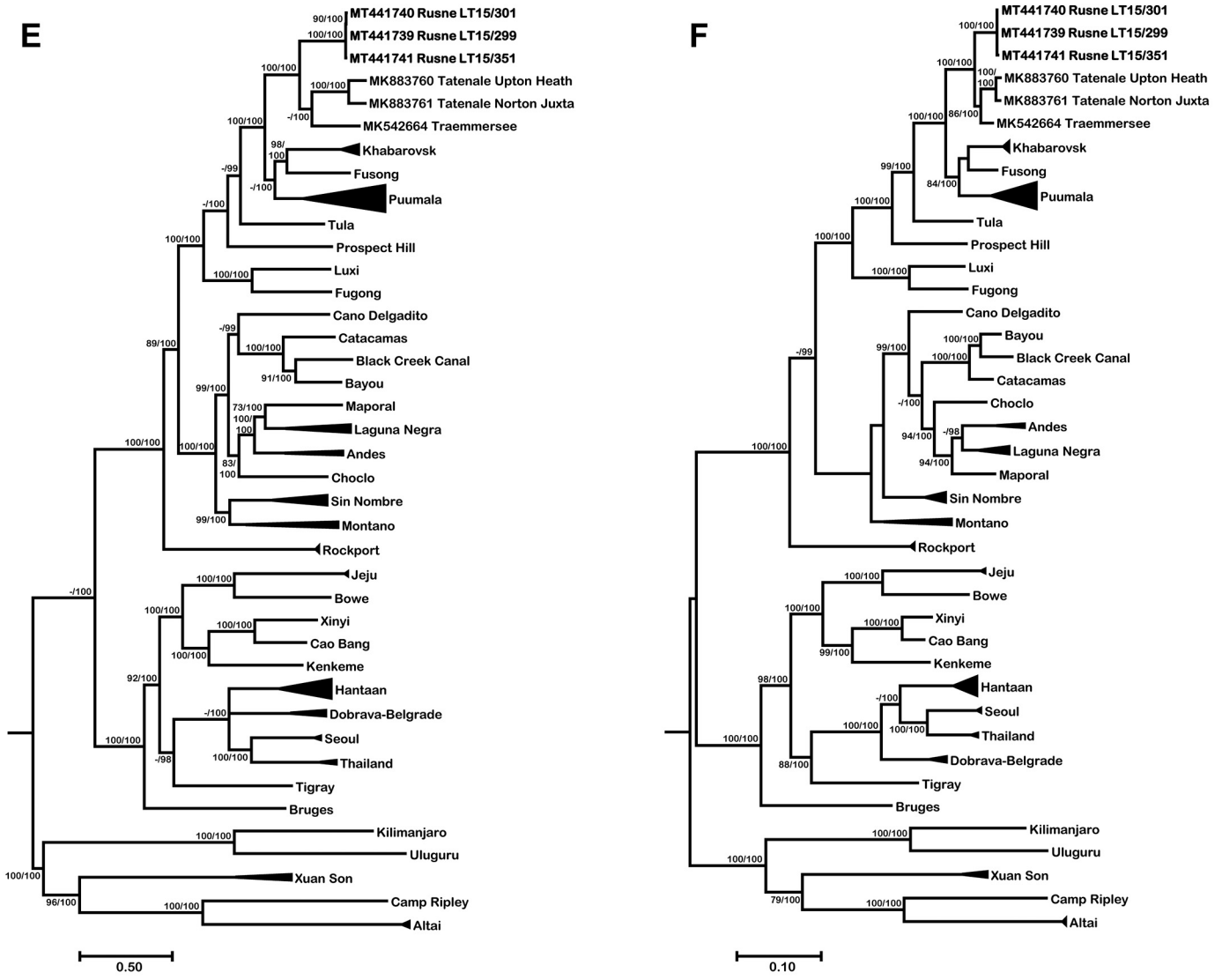

Fig. 3.. (*continued*)

Writing - review & editing, Project administration, Funding acquisition. Rainer G. Ulrich: Conceptualization, Writing - original draft, Supervision, Writing - review & editing, Project administration.

Ethical statement

Rodent sampling was conducted with permission from the Environmental Protection Agency (EPA) and approved by the Ministry of Environment of the Republic of Lithuania, licenses No. 22 (2015-04-10), No. 12 (2016-03-30), No 13 (2017-03-22) and No 6 (2018-02-02) in accordance with Lithuanian (the Republic of Lithuania Law on Welfare and Protection of Animals No. XI-2271) and European legislation (Directive 2010/63/EU) on the protection of animals.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104520.

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