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Faculty of Science

Orthohantaviruses in the reservoir and atypical hosts in the Czech Republic: spillover infection and indication of virus-specific tissue tropism

RNDr. Thesis

Mgr. Jan Kamiš

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Annotation:

Aim of this study was to reveal the presence of hantaviruses in natural reservoir rodent hosts in selected urban areas in the Czech Republic. Hantavirus rodent hosts were trapped, sampled and tested for hantavirus RNA in different tissues. Universal and specific primers for amplification of the large and medium fragments of hantavirus genomic RNA were used. Phylogenetic relationships were based on the obtained nucleotide sequences. Four different hantaviruses were detected, including two species pathogenic (or potentially pathogenic) for humans, further suggesting a threat for public health. Moreover, inter-family spillover infections and hantavirus species-associated tissue tropism were recorded in rodent hosts.

DECLARATION

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 7.9.2022

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Mgr. Jan Kamiš

Orthohantaviruses in the reservoir and atypical hosts in the Czech Republic: spillover infection and indication of virus-specific tissue tropism

Running title: Orthohantaviruses in reservoir and spillover hosts

Václav Hönig [a, b*¶], Jan Kamiš[a, c¶], Aneta Maršíková[c], Tereza Matějková[d], Pavel Stopka[d], Anna Mácová[c], Daniel Růžek[a, b, e], Jana Kvičerová [c, d]

[a]Laboratory of Arbovirology, Institute of Parasitology, Biology Centre CAS, Branišovská 31, České Budějovice 37005, Czech Republic

[b]Laboratory of Emerging Viral Infections, Veterinary Research Institute, Hudcova 70, Brno 62100, Czech Republic

[c]Department of Parasitology, Faculty of Science, University of South Bohemia, Branišovská 1760, České Budějovice 37005, Czech Republic

[d]Department of Zoology, Faculty of Science, Charles University, BIOCEV, Průmyslová 595, Vestec 25250, Czech Republic

[e]Department of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 735/5, Brno 62500, Czech Republic

[*]Corresponding author: honig@paru.cas.cz (VH)

[¶]These authors contributed equally to this work.

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ABSTRACT

Orthohantaviruses (genus *Orthohantavirus*) are a diverse group of viruses that are closely associated with their natural hosts (rodents, shrews, and moles). Several orthohantaviruses cause severe disease in humans. Central and Western Europe are areas with emerging orthohantavirus occurrence. In our study, several orthohantaviruses, including the pathogenic Kurkino virus (KURV), were detected in their natural hosts trapped at several study sites in the Czech Republic. KURV was detected mainly in its typical host, the striped field mouse (*Apodemus agrarius*). Nevertheless, spillover infection was also detected in wood mice (*A. sylvaticus*) and common voles (*Microtus arvalis*). Similarly, Tula virus (TULV) was found primarily in common voles, and spillover events to rodents of other host species including *Apodemus* spp. were recorded. In addition, unlike most previous studies, different tissues were sampled and compared to assess their suitability for orthohantavirus screening, and possible tissue tropism. Our data suggest possible virus-specific tissue tropism in rodent hosts. TULV was most commonly detected in the lung tissue, whereas KURV was more common in the liver, spleen, and the brain. Moreover, Seewis and Asikkala viruses were detected in randomly found common shrews (*Sorex araneus*). In conclusion, we have demonstrated the presence of human pathogenic KURV and the potentially pathogenic TULV in their typical hosts as well as their spillover to atypical host species belonging to another family. Furthermore, we suggest the possibility of virus-specific tissue tropism of orthohantaviruses in their natural hosts.

Importance

Orthohantaviruses (genus *Orthohantavirus*, family *Hantaviridae*) are a diverse group of globally distributed viruses that are closely associated with their natural hosts. Some orthohantaviruses are capable of infecting humans and causing severe disease. Orthohantaviruses are considered emerging pathogens due to their ever-increasing diversity and increasing number of disease cases. We report detection of four different orthohantaviruses in rodents and shrews in the Czech Republic. Most viruses were found in their typical hosts, Kurkino virus (KURV) in striped field mice (*Apodemus agrarius*), Tula virus (TULV) in common voles (*Microtus arvalis*), and the Seewis virus in common shrews (*Sorex araneus*). Nevertheless, spillover infections to atypical host species were also recorded for KURV, TULV and another shrew-borne orthohantavirus, Asikkala virus. In addition, indications of virus-specific patterns of tissue tropism were observed. Our results highlight the circulation of several

orthohantaviruses, including KURV, which is pathogenic to humans, among rodents and shrews in the Czech Republic.

INTRODUCTION

Orthohantaviruses (genus *Orthohantavirus*, family *Hantaviridae*, order *Bunyavirales*) are negative-sense, enveloped, single-stranded zoonotic RNA viruses with a tri-segmented genome (formed by large - L, medium - M, and small - S segments) (1, 2). In humans, they may cause infection with two types of clinical manifestation, both with possible fatal outcome (3, 4). Hemorrhagic fever with renal syndrome, HFRS, is caused by Old World orthohantaviruses that occur in Europe and Asia, whereas hantavirus pulmonary (or cardiopulmonary) syndrome, H(C)PS, is caused by New World orthohantaviruses in the Americas (5, 6). Orthohantaviruses are considered host-specific and are closely tightly associated with hosts of one or few closely related species that constitute their natural reservoir (6–9). The reservoir hosts of orthohantaviruses pathogenic for human are rodents, but other orthohantaviruses have also been detected in Eulipotyphla (namely shrews and moles) (10, 11). As rodents are widespread and people can easily come into contact with them, human infections have become an increasing problem. Inhalation of virus-containing aerosols via the excreta (urine, faeces, or saliva) of infected rodents is the most common route of transmission (10, 12).

In general, orthohantaviruses form three large evolutionary groups (as numbered below) associated with hosts from four rodent subfamilies, including 1. Old-World subfamilies Murinae (family Muridae), 2. Arvicolinae (family Cricetidae), and 3. New-World subfamilies Sigmodontinae (Cricetidae) and Neotominae (Cricetidae) (8, 13). In addition, some orthohantaviruses are associated with hosts of the order Eulipotyphla (families Soricidae, Talpidae) as their reservoir hosts (13). In Europe, the following orthohantaviruses circulate in populations of wild rodents: Dobrava virus (DOBV), Kurkino virus (KURV), Saaremaa virus (SAAV), Sochi virus (SOCV) (all belonging to *Dobrava-Belgrade orthohantavirus* species), Puumala virus (PUUV; *Puumala orthohantavirus*), Seoul virus (SEOV; *Seoul orthohantavirus*), and Tula virus (TULV; *Tula orthohantavirus*) (8, 14–17). Moreover, Seewis virus (SWSV; *Seewis orthohantavirus)* and Asikkala virus (ASIV, *Asikkala orthohantavirus*) have been found mainly in shrews (18, 19). Most of the European orthohantavirus human disease cases are caused by PUUV, DOBV, and KURV (20). The viruses differ in their geographic distribution, species of reservoir hosts, and virulence to humans. DOBV (previously known as DOBV-Af), typically hosted by yellow-necked mice (*Apodemus flavicollis*, Murinae), is dominant in the Balkans and Russia (21). It has also been found in several countries in Central Europe (e.g. in the Czech Republic, Germany, Hungary, or Slovakia) (8, 21, 22). KURV (previously known as DOBV-Aa) is associated with striped field mice (*A. agrarius*) and

is widely distributed from Germany throughout the Central European countries to parts of northern (Denmark) and eastern (Estonia, Russia) Europe, and causes a milder form of human disease compared with DOBV (8, 23, 24). Striped field mice are also the reservoir hosts of SAAV, so far restricted to the island of Saaremaa in Estonia (7). SOCV (previously known as DOBV-Ap) is associated with Black Sea field mice (*A. ponticus*), and occurs in the Black Sea region of the European part of Russia (7, 25). More common but less virulent PUUV is the causative agent of a HFRS-like disease called *nephropathia epidemica* (NE) (3). Together with its reservoir host, bank vole (*Clethrionomys glareolus*, Arvicolinae), it is distributed throughout Europe and in the western part of Russia (23, 26). Furthermore, the simultaneous co-occurrence of PUUV, DOBV, and KURV in the same area has been reported, particularly in the Balkans (27). SEOV, which is transmitted by rats (*Rattus* spp., Murinae) is an exceptional orthohantavirus, that is distributed worldwide due to ship trade and human migration, allowing the movement of rats over long distances (26, 28). TULV is found primarily in common voles (*Microtus arvalis*, Arvicolinae), several other members of the same genus, and European water voles (*Arvicola amphibius*, Arvicolinae) (29–31). Although TULV is considered non-pathogenic, rare cases of TULV-associated pulmonary and renal syndrome have been documented in humans in the Czech Republic and Germany (32, 33).

Regarding shrew-borne orthohantaviruses, SWSV was first detected in a common shrew (*Sorex araneus*, Soricidae) captured in an Swiss village of the same name (34). Since then, several studies have confirmed SWSV in shrews and occasionally also in rodents in other Central European countries including the Czech Republic, Slovakia, and Germany (19, 35). Another shrew-borne hantavirus, ASIV, has been recorded as a novel hantavirus from Finland (36), carried by the Eurasian pygmy shrew (*S. minutus*). Together with SWSV, ASIV has also been detected in the Czech Republic and neighbouring Germany (18).

Although orthohantaviruses are not new to mankind, they are considered to be emerging viruses with epidemic outbreaks because of the recent increase in number of human cases (especially in Western Europe) (37) and because of the continuing records of enormous previously unrecognized diversity (5, 7, 38, 39). In contrast to the observed seroprevalence (22), the incidence of orthohantavirus infection in humans is lower in the Czech Republic than in neighbouring Germany or Austria (20, 40). Data on the circulation of orthohantaviruses among reservoir hosts are incomplete, yet human cases and rodent tissue screening of suggest the presence and epidemiologic relevance of DOBV, KURV, PUUV, and TULV (35) in this country. Here we report KURV and TULV, their phylogenetic relationships, and their

occurrence in different host tissues of wild rodents mainly from urban areas of the Czech Republic, as well as SWSV and ASIV in randomly found shrews.

MATERIALS AND METHODS

Ethical statements

This study included trapping of free-living rodents. The trapping and manipulation with the trapped animals were carried out in a strict accordance with the Czech national laws and guidelines on the use of experimental animals and protection of animals against cruelty (Animal Welfare Act No. 246/1992 Coll.). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia, and by the Ministry of the Environment of the Czech Republic (Permit Numbers 51304/ENV/14-2981/630/14, MZP/2017/630/854, and MZP/2021/630/2459).

Sampling

In the course of 2016-2021, rodents (yellow-necked field mice, striped field mice, wood mice, common voles, and bank voles) were live-trapped in 14 areas of the Czech Republic (Table 1, Fig. 1). Furthermore, randomly found cadavers of shrews (10 individuals) were collected and also subjected to the screening process (Table 2, Fig. 1).

Sherman-live traps (LFA size; H.B. Sherman Traps, Inc., Tallahassee, FL, USA) filled with bait were set in the late evening, spaced approximately 10 meters apart and left in the field overnight. The lungs and occasionally also other visceral organs: liver, kidneys, spleen, brain and heart were sampled directly after the animal was killed by cervical dislocation, and preserved in RNA stabilization solution (RNAlater, Invitrogen, Vilnius, Lithuania). Sterile dissection tools were used for each individual and cleansed between sampling of the individual organs. After the transportation to the laboratory, the samples were stored at −80 °C. Detailed data on individual rodents are presented in Supplementary Table S1.

Reservoir hosts of species with overlapping morphologies that are difficult to be distinguished in the field (yellow-necked field mice, wood mice, and shrews) were identified by methods of molecular biology (diagnostic PCR and sequencing) (41, 42).

150 ***RNA extraction and reverse transcription***

151 Individual rodent tissue samples were cleansed from the RNA later, and homogenized in sterile
152 phosphate-buffered saline (PBS) as 10% (liver) or 20% (all remaining tissue samples)
153 suspensions (w/v) using an automated homogenizer (Tissue Lyzer II, Qiagen, Hilden,
154 Germany) and sterile 5 mm stainless-steel beads at 30 Hz for 2 min (Qiagen, Hilden, Germany).
155 After centrifugation the supernatant was collected and RNA isolation was performed using a
156 commercially available silica column-based kit (QIAamp Viral RNA Mini Kit, Qiagen, Hilden,
157 Germany) according to the manufacturer's instructions. Using High-Capacity RNA-to-cDNA
158 Kit (Thermo Fisher Scientific, Vilnius, Lithuania) and 5 µl of total RNA as template, cDNA
159 was synthesized according to the manufacturer's instructions.

160 ***PCR amplification and sequencing***

161 *Screening PCR*

162 All the available samples were screened for orthohantavirus RNA. A nested PCR with Han-L-
163 F1 + Han-L-R1 (first reaction), and Han-L-F2 + Han-L-R2 (second reaction) primers (Table 3)
164 was used to amplify the partial sequences of the orthohantaviral L segment encoding the RNA-
165 dependent RNA polymerase (43) . The first PCR was carried out in the total volume of 25 µl,
166 including 1.0 µl of each primer (10 µM), 12.5 µl of PCR master mix (Combi PPP Master Mix,
167 Top-Bio, s.r.o., Vestec, Czech Republic), 6.5 µl of PCR water, and 4 µl of synthesized cDNA.
168 Annealing temperature was set based on the best result of the gradient PCR. Parameters for
169 nested PCR reactions were as follows: initial denaturation at 95 °C for 6 min, followed by 40
170 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for
171 30 s. The final extension step was performed at 72 °C for 3 min. Subsequently, 1 µl of the
172 product of the first PCR was used for the nested reaction following the same protocol (the
173 missing volume in the PCR reaction was filled with PCR water). Individual steps of the
174 detection protocol (nucleic acid extraction, preparation of PCR mastermixes, amplification,
175 electrophoresis and PCR product purification) were performed in separate rooms, using
176 separate equipment. Moreover, PCR mastermixes were prepared in a dedicated PCR box,
177 samples and isolated nucleic acids were handled in biohazard boxes, all working surfaces were
178 before and after the work decontaminated using bleach and UV light.

M segment-specific PCR

Samples positive for RNA of the viruses belonging to *Dobrava-Belgrade orthohantavirus* species (according to the sequencing of the screening PCR product) were submitted to amplification of the partial sequence of the orthohantaviral M segment encoding the Gn and Gc glycoprotein precursors. The PCR reactions were prepared as described for the screening nested PCR, employing the 1470c, 2029R (first PCR) and 1674F, 1990R (second PCR) primer pairs (16) (Table 3). The parameters for the PCR reaction were as follows: initial denaturation at 95 °C for 6 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 40 °C for 30 s, and extension at 72 °C for 30 s. The final extension step was performed at 72 °C for 3 min. Primer pair 28F, 492R (Table 3) was used for TULV-positive samples, following the previously described protocol and parameters with the exception of the annealing temperature at 50 °C.

Processing of the PCR products and sequencing

PCR amplicons were visualized on 2% agarose gel using Sybr Green (Life technologies Europe, Bleiswijk, the Netherlands) under UV light (UVITEC, Cambridge). PCR products of expected sizes were purified using 0.2 µl of FastAP (Thermosensitive Alkaline Phosphatase) and 0.2 µl of Exo I (Exonuclease I from *E. coli*) enzymes (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Enzymatic digestion was carried out in a thermocycler at 37 °C for 15 min followed by enzyme inactivation at 80 °C for 15 min. Purified PCR products were directly sequenced via the Sanger sequencing method in Macrogen, Inc. (Amsterdam, the Netherlands) on an automatic 3730XL DNA analyzer (http://www.macrogen.com/eng/business/seq_service.html). Obtained sequences were verified by the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and adjusted in Sequence Scanner v2.0 (https://products.appliedbiosystems.com). Programs EditSeq and SeqMan v5.05 (DNASTAR Inc., Madison, Wisconsin, USA) were used to assemble the sequences. The sequences were then deposited in the NCBI GenBank database (www.ncbi.nlm.nih.gov) under the accession numbers ON243777-243817; ON653425-ON653442 (Supplementary Table S2, S3).

Phylogenetic analyses

The obtained partial sequences of the L and M genomic segments of orthohantaviruses from rodents and shrews, together with the sequences of related orthohantaviruses available in the GenBank database, were used for phylogenetic analyses. The dataset was aligned in the program BioEdit v7.2.5 (44) using the ClustalW Multiple Alignment (45) algorithm. The resultant alignment was manually trimmed to the uniform length. For the reconstruction of phylogenetic relationships, two approaches were used: Bayesian inference (BI) performed in MrBayes v3.2.2 (46), and maximum likelihood (ML) in PhyML v2.4.3 (47). The most suitable evolutionary models were selected by jModeltest (48, 49). BI analysis was calculated under the GTR + Γ + I evolutionary model; MCMC was specified for 10 million generations with a frequency of collection every 500 generations, and burn-in was set to 25 %. ML was also conducted using the GTR + Γ + I model, and bootstrap values were calculated by 1,000 replicates. The resultant phylogenetic trees were visualized and exported in TreeView v1.6.6 (50), and graphically edited in Adobe Illustrator CC v2017.0.2 (Adobe Systems, Inc.).

Statistical analyses

Differences in orthohantavirus prevalence between female and male hosts as well as differences in the prevalence of particular orthohantavirus species in the individual tissues were tested using the Fisher's exact test (GraphPad Prism v9.3.1, GraphPad Software, CA, USA). Differences with $p<0.05$ were considered statistically significant.

RESULTS

Altogether, 153 rodent individuals were trapped and sampled at the defined trapping sites (for details, see Table 4). Moreover, 10 randomly found dead shrews (family Soricidae: *Sorex* spp., *Crocidura* spp., *Neomys fodiens*) were also sampled (Table 2).

Prevalence and diversity of detected orthohantaviruses

In total, 24.2 % (37/153) of the rodent hosts and 27.3 % (3/10) of the shrews tested positive for orthohantavirus RNA (PCR products confirmed by sequencing) in at least one tissue sample (multiple tissue samples were taken from a trapped individual). Based on the nucleotide sequence analysis, TULV, KURV, SWSV and ASIV were identified in the positive samples. TULV was most frequently found in common voles (70.8 % of all trapped common voles) and

KURV in striped field mice (15.2% of all trapped striped field mice), even though both viruses were also detected in rodents of other species (Table 5). SWSV and ASIV were found exclusively in common shrews. Differences in prevalence rate between the female and male hosts were not statistically significant neither on the level of localities, nor the level of the individual host species (detailed results in Supplementary Table S3).

Phylogenetic analyses

The final alignment of L segment sequences yielded a 290 bp long matrix containing 97 sequences of orthohantaviruses; the final alignment of M segment sequences was 292 bp long and contained 39 sequences of orthohantaviruses. Phylogenetic analyses of both matrices produced well-resolved trees with a basic structure corresponding to the phylogenies presented in Klempa et al. (7) and Zelená et al. (35). However, the addition of the DOBV, KURV, TULV, SWSV, ASIV, and other orthohantaviruses into the common phylogeny has made the overall evolutionary picture within the genus *Orthohantavirus* even more complex.

All 9 KURV sequences of L segment obtained from our samples which originated from striped field mice (6 sequences), common voles (2 sequences), and a yellow-necked mouse (1 sequence) were placed to the KURV branch. They were split into two distinct clusters regardless of the host species, locality, or tissue (Fig. 2). Of the M segment, we managed to obtain only a single sequence from samples previously positive for KURV (according to the L segment sequence). That sequence was obtained from a striped field mouse and could not be assigned to a specific virus clade as the whole *Dobrava-Belgrade orthohantavirus* cluster remained unresolved in the M segment tree (Fig. 3).

We obtained 28 TULV sequences of L segment which originated from common voles (18 sequences), striped field mice (5 sequences), bank voles (2 sequences), wood mice (2 sequences), and a yellow-necked mouse (1 sequence). They branched within two phylogenetically distinct clusters based on the sampled localities. One of the branches was almost exclusively associated with samples from Vestec (Fig. 2). Less TULV sequences were obtained for the M segment (18 sequences), but still indicating the same pattern of the two distinct clusters (Fig. 3).

Two sequences of L segment from common shrew clustered with SWSV sequences, while one sequence represented ASIV. Unfortunately, we did not manage to sequence the M segment of any samples from shrews, despite multiple efforts.

Tissue tropism

Concerning the tissue specificity and efficiency of orthohantavirus RNA detection, virus-specific patterns were observed. TULV was most efficiently detected in the lung tissue (82% of the individuals positive in any tissue), whereas KURV was more efficiently detected in the liver (71%) and the spleen (71%), and most surprisingly in the brain (75%) (Table 6). No TULV-positive kidney samples were found in the tested mice nor in bank voles including 6 samples of individuals positive in other tissues, whereas the same virus was efficiently detected in the kidney tissue of 65% of the positive common voles (Supplementary table S4). Nevertheless, the differences in the prevalence of TULV and DOBV in the individual tissue samples were not statistically significant. Shrew-borne orthohantaviruses were found in the lungs, liver, brain and the heart tissue (Supplementary Table S4).

DISCUSSION

Orthohantaviruses are emerging zoonotic pathogens that have a significant impact on human health in many countries (51). Although a similar or even higher seroprevalence has been found in the human population in the Czech Republic, the incidence rate of orthohantavirus human cases is significantly lower compared with other countries in Central Europe, especially compared with neighbouring Austria, Germany, and Slovakia (52). This could be due to an underestimation of the number of clinical cases or by a higher occurrence of clinically inapparent cases or (most likely) a combination of both. KURV and TULV are among the most frequently detected orthohantaviruses in rodents in the Czech Republic, both in our (Table 5) and in previous studies (29, 35). Both pathogens are associated with a mild course of the disease (53, 54). In contrast, PUUV has been reported as a major cause of human infection elsewhere in Europe (55), and also in Austria (56) and Germany (53), including areas bordering the Czech Republic. DOBV and KURV human HFRS cases are significantly less frequent in Central Europe (53, 57). In the Czech Republic, PUUV, DOBV, and KURV are the most frequent causes of clinically apparent, diagnosed orthohantavirus disease cases in humans (16, 35, 58, 59), although they remain relatively rare and spatially and geographically isolated.

KURV was detected mainly in striped field mice, two wood mice, and two common voles (Table 5). The presence of the related DOBV was previously reported in 2 yellow-necked mice in Northern Moravia (35) and in rodents of multiple species in South Bohemia (60). Interestingly, in our study, KURV was detected in multiple individuals at the two trapping sites in Northern Moravia and one trapping site in South Bohemia (Fig. 1). The obtained nucleotide

sequences from both regions clustered together with sequences from rodents and human patients from Northern Moravia (35). The authors of the previous study (35) mentioned that DOBV was more frequently detected in mountainous areas, whereas KURV was associated with lowlands; our samples originated from lowlands.

In our study, PUUV was not detected in any of 20 bank voles or in animals of any other species. There is a single study reporting direct detection of PUUV in rodents in the Czech Republic (59), indicating that the distribution of this virus might be highly focal. As also previously reported (29, 61, 62), TULV is prevalent among populations of common voles in the Czech Republic. Although it is rarely detected in humans, infections of immunocompromised (33) as well as immunocompetent patients were reported (32, 57, 63). In general, the distribution of orthohantaviruses in their reservoir hosts, as well as the distribution of human cases, is influenced by numerous factors on the side of the reservoirs, the virus, and the human population (52, 64), resulting in a high spatio-temporal variability (53).

Phylogenetic analyses of the L segment indicate that the detected TULV, and shrew-borne orthohantaviruses are strictly monophyletic. The members of *Dobrava-Belgrade orthohantavirus* species split into 4 monophyletic lineages according to the individual viruses, DOBV, KURV, SAAV, and SOCV, which is in congruence with the former publications of Klempa et al. (7) and Zelená et al. (35). Our sequences were classified as KURV. Similarly, it seems obvious that TULV is not composed of a single genotype, but it also splits in several distinct genotypes within the Central Europe, regardless their reservoir host (53, 65, 66). Since only a little is known of its pathogenicity to humans, we cannot assess whether this differentiation may have any significance in the terms of impact on human health (i.e. that one lineage may be more pathogenic than the other). Phylogenetic analyses of the M segment were congruent with results of Klempa et al. (7) suggesting that the phylogenetic position of SAAV is unresolved, being scattered among the viruses of *Dobrava-Belgrade orthohantavirus* species. The phylogram of the M segment was less resolved compared to the L segment. The M segment, encoding the Gn and Gc surface glycoprotein precursors, is known to undergo faster evolution compared to the L (RNA-dependent RNA polymerase) and S (nucleocapsid) segments (67, 68), which is reflected in the long branch of TULV in the M segment compared to L segment phylogenetic tree.

Orthohantaviruses are considered to be highly host-specific (8, 69). In our study, the majority of TULV was detected in common voles (family Cricetidae), which are typical hosts of the virus in Central Europe (29, 30). Similarly, as expected, KURV was most frequently found in striped field mouse (Muridae) (7), and SWSV and ASIV were detected exclusively in

common shrews (18, 34). Nevertheless, TULV RNA was detected in four striped field mice, two wood mice, two yellow-necked mice and two bank voles , and likewise two wood mice and one common vole were positive for KURV RNA. Most of the atypical hosts shared the same locality (i.e., lived syntopically) with the positive individuals of the typical host species, and the sequence analysis confirmed high identity of sequences obtained from typical and atypical hosts, indicating an inter-species (inter-family) spillover. The possibility of cross-contamination can never be completely eliminated, but we have taken measures to minimize this risk. In addition, the virus was detected in multiple tissues from the same individual infected with an atypical orthohantavirus and the individuals originated from different trapping sites and trapping events which makes an accidental cross-contamination highly unlikely. The possibility of infection of bank voles with TULV as well as infection of mice (yellow-necked mice and laboratory mice) with atypical viruses of the *Dobrava-Belgrade orthohantavirus* species was partially confirmed in a laboratory experiment (70). There is evidence that spillover infection between different species occur under natural conditions between the host species belonging to the same family (60, 66, 71, 72) rather than between members of different families (35, 60). However, exclusive use of the typical host even in the conditions of sympatric/syntopic occurrence of the hosts and viruses has also been reported (4, 73). On the other hand, surveillance of hantaviruses often focuses on a particular host species and/or particular virus, therefore, the frequency of inter-genus spillover may be underestimated. Our data do not allow us to assess whether infection of an atypical host results in the same course of infection and whether and how effectively atypical hosts may participate in virus circulation in nature. Nevertheless, our records of KURV and TULV hantavirus spillover to hosts of different families indicate possible lower host specificity and potential for hantavirus co-infections. Interestingly, one striped field mouse (52AA, only short KURV sequence available – not included in the phylogenetic analysis) and one common vole (23723MA) were found to be infected simultaneously by KURV and TULV (Fig. 2). Although each of the viruses was detected in a different organ, such a co-infection can lead to reassortment or recombination events (39) because the two viruses may encounter each other in the same tissue at a different stage of infection.

Orthohantaviruses, as viruses with a segmented genome, may exchange the segments and form reassortants. Unlike orthobunyaviruses, they usually form reassortants within members of the same virus or virus species rather than between two different virus species. The M segment is most likely to be replaced, while the combination of L and S segments usually remains stable (39). The evidence of reassortments is usually revealed as a conflicting topology

368 of virus nucleotide sequences of each genomic segment from the same host individual.
369 Therefore, we compared the phylogenetic position of the L segment sequences to their position
370 in the M segment phylogenetic tree (Fig. 2, 3). No evidence of inter-species reassortment was
371 found. Nevertheless, while one TULV sequence obtained from common vole trapped in Praha-
372 západ district (4MI) grouped together with all other sequences from the same locality in the L
373 segment-based phylogenetic tree (Fig. 1), its position in the M segment-based phylogeny
374 indicates possible reassortment between two TULV lineages (Fig. 2). However, because only
375 short sequences of both genome fragments were available, we are not able to distinguish
376 between reassortment and homologous recombination (39).
377 Most studies on trapped rodents have screened only a single tissue, usually the lungs
378 (21, 35, 59) or the kidneys (73) for orthohantavirus detection. Because there might be
379 differences in the efficiency of orthohantavirus detection in different tissues, we compared the
380 detection rate of TULV and KURV in positive individuals in all different available tissues.
381 Although the differences were not statistically significant (possibly because of the insufficient
382 number of positive samples and incomplete tissue sample set of several individuals
383 (Supplementary table S1)), our results generally confirmed the observations from the previous
384 studies: namely, a lower detection efficiency of KURV (DOBV) compared with TULV in the
385 lungs, a high efficiency of orthohantavirus detection in the liver, and the possibility to detect
386 orthohantaviral RNA in brain tissues of rodents and shrews (Supplementary table S4) (15, 66,
387 74, 75). Based on our results, we hypothesize that the tissue tropism is virus-specific not only
388 in humans, but also in natural orthohantavirus rodent hosts and that infection is often
389 multisystemic. These observations need to be confirmed on a larger scale and with a complete
390 sample set that would allow adequate statistical evaluation. Nevertheless, our pilot findings are
391 of great importance because these mechanisms may significantly affect the overall efficiency
392 of orthohantaviral RNA detection.
393 In addition to from rodent-associated orthohantaviruses, RNA of shrew-borne
394 orthohantaviruses SWSV and ASIV was also detected in our study. Considering the fact that
395 the shrews were found completely randomly at different, geographically distant locations, and
396 yet 3 out of 10 were positive for orthohantavirus RNA (only common shrews), we assume a
397 high prevalence of these orthohantaviruses in shrews in the Czech Republic. SWSV has already
398 been detected several times in Central Europe (34, 76), and particularly in the Czech Republic
399 (31, 35). Our L segment sequences obtained from common shrews formed a well-supported
400 separate intra-cluster within the SWSV clade. It is evident that all three sequences from the
401 Czech Republic are distinct from those from Slovakia, Russia, and Finland (19, 77). The L

segment SWSV sequence JQ425313 (19), from a common shrew in the GenBank database originates from the same district, České Budějovice, where we detected the SWSV-positive sample 5SA. Concerning the time gap between the finding of the two positive individuals of common shrews (11 years) and 99% L segment nucleotide identity (328/330), we can state that after all these years, SWSV in České Budějovice is still present and circulates in shrews in this area almost unchanged. We also detected ASIV in another common shrew (sample 4SA). ASIV was detected in the Czech Republic and neighbouring Germany both in common shrews and Eurasian pygmy shrews. Sympatric occurrence of these species provides an opportunity for spillover infections, however, phylogenetic analyses and broad geographical distribution of ASIV across Europe in Eurasian pygmy shrews imply shrews of this species as the primary reservoir hosts (18).

In conclusion, we detected multiple orthohantaviruses in free-living rodents and shrews in the Czech Republic. Moreover, our data suggest possible virus-specific tissue tropism in rodent hosts, high prevalence of SWSV in common shrews and high prevalence of TULV in common voles (with frequent spillover to hosts of other species including Muridae) in the Czech Republic. Since most of the rodents were trapped in close vicinity of human settlements, and human pathogenic KURV and potentially pathogenic TULV were found, our results suggest a potential risk to public health.

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Disclosure statement

The authors report there are no competing interests to declare.

Author's contributions

VH: conceptualization, formal analysis, methodology, project administration, supervision, validation, original draft preparation, writing – review and editing

JK: data curation, formal analysis, investigation, methodology, resources, visualization, writing – original draft preparation, writing – review and editing

AM: investigation, methodology, resources, original draft preparation

TM: investigation, resources

PS: investigation, resources, funding acquisition

AnMa: investigation, resources

DR: funding acquisition, supervision, writing – review and editing

JaKv: conceptualization, data curation, formal analysis, funding acquisition, methodology, resources, supervision, validation, original draft preparation, writing – review and editing

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693 Table 1. Detailed information on the localities of rodent trapping.

Locality code	Locality; District (Region)	Character of the locality	GPS coordinates (WGS84)	Year of collection
1	Borek; České Budějovice (South Bohemia)	urban area	49°00'45.677"N, 14°29'46.141"E	2016
2	Vltava; České Budějovice (South Bohemia)	urban area (housing estate)	48°59'56.238"N, 14°27'19.339"E	2017
3	Mánesova street no. 273/9; České Budějovice (South Bohemia)	urban area (house cellar)	48°58'09.730"N, 14°28'45.020"E	2018
4	Švábův Hrádek; České Budějovice (South Bohemia)	rural area (weed)	48°58'16.600"N, 14°26'20.212"E	2020
5	Lužnice, field station U Zahradníků no. 92; Jindřichův Hradec (South Bohemia)	rural area (congress centre)	49°04'51.428"N, 14°45'41.266"E	2018
6	Zbytiny – Koryto; Prachatice (South Bohemia)	area of confirmed hantavirus disease in man	48°55'53.899"N, 14°01'23.761"E	2018
7	Květušín; Český Krumlov (South Bohemia)	area of confirmed hantavirus disease in man	48°46'56.620"N, 14°07'59.710"E	2021
8	Oldřišov; Opava (Northern Moravia)	rural area (agricultural)	49°58'36.249"N, 17°57'30.491"E	2016
9	Oldřišov, sugar beet field between Oldřišov and Opava; Opava (Northern Moravia)	rural area (agricultural)	49°59'04.414"N, 17°56'47.773"E	2016
10	Weed hill near the Hillova street; Opava (Northern Moravia)	urban area	49°57'11.994"N, 17°54'55.937"E	2016
11	Varnsdorf; Děčín (Northern Bohemia)	rural area (agricultural)	50°55'09.899"N, 14°35'53.808"E	2018, 2019
12	Vestec, Biocev; Praha-západ (Central Bohemia)	urban area (research center complex)	49°58'54.020"N, 14°29'16.572"E	2020
13	Vestec, near the Shell gas station; Praha-západ (Central Bohemia)	urban area	49°59'34.318"N, 14°29'32.185"E	2020
14	Dolní Břežany; Praha-západ (Central Bohemia)	urban area	49°57'44.389"N, 14°27'57.209"E	2020

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696 Table 2. Detailed information on the randomly found dead shrews.
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Locality code	Name of the locality (District)	Character of the locality	GPS coordinates (WGS84)	Year of collection	Species of collected animal
A	České Budějovice, Vltava (České Budějovice)	urban area (housing estate)	48°59'56.238"N, 14°27'19.339"E	2017	*Sorex minutus*
B	České Budějovice, Biology Centre CAS (České Budějovice)	urban area (research center complex)	48°58'39.859"N, 14°26'52.175"E	2020	*Sorex araneus*
C	Zbytiny - Koryto (Prachatice)	area of confirmed hantavirus disease in man	48°55'53.899"N, 14°01'23.761"E	2018	*Sorex araneus*
D	Volenice (Strakonice)	rural area (agricultural)	49°32'26.700"N, 13°54'06.000"E	2019	*Crocidura suaveolens*
E	Lužnice, field station U Zahradníků no. 92 (Jindřichův Hradec)	rural area (congress center)	49°04'51.428"N, 14°45'41.266"E	2018	*Neomys fodiens* (N=2)
F	Hoděmyšl (Příbram)	urban area	49°36'41.220"N, 13°53'17.700"E	2019	*Crocidura suaveolens*
G	Podmokly (Plzeň-sever)	rural area (agricultural)	49°52'04.020"N, 13°10'00.240"E	2019	*Sorex araneus*
H	Varnsdorf (Děčín)	rural area (agricultural)	50°55'09.899"N, 14°35'53.808"E	2018	*Sorex araneus*
I	Semtěš (Karlovy Vary)	rural area (agricultural)	50°04'32.460"N, 13°09'41.700"E	2019	*Crocidura leucodon*

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Table 3. Primers used for the screening of rodent tissue samples and sequencing of orthohantavirus-positive samples.

Primer name	Sequence	Sense	Annealing temperature [°C]	Approx. size of PCR product [bp]	Target	Reference
HAN-L-F1	ATGTAYGTBAGTGCWGATGC	forward (F)	53	420	L segment	(43)
HAN-L-R1	AACCADTCWGTYCCRTCATC	reverse (R)				
HAN-L-F2	TGCWGATGCHACIAARTGGTC	F	53	390		
HAN-L-R2	GCRTCRTCWGARTGRTGDGCAA	R				
1470c	CCIGGITTICATGGITGGGC	F	40	600	M segment DOBV	(16)
2029R	CCATGIGCITTITCIKTCCA	R				
1674F	TGTGAIKTITGIAAITAIGAGTGTGA	F	40	320		
1990R	TCIGMTGCISTIGCIGCCCA	R				
28F	AATTGAAAAGGTGAAGCAGG	F	50	460	M segment TULV	this study
492R	GCAGATGATGGTAGGGAAAA	R				

705 Table 4: Summary of the number and species of the trapped and examined rodents in the
706 Czech Republic during the years 2016-2021[a].

Locality (Region)	Trapping date	MA	CG	AA	AS	AF
České Budějovice (South Bohemia)	2016-2018	4	7	-	12	15
Lužnice (South Bohemia)	2018	-	10	-	-	1
Zbytiny – Koryto (South Bohemia)	2021	-	2	-	5	-
Květušín (South Bohemia)	2021	2	-	-	1	2
Opava (Northern Moravia)	2016	1	-	40	1	10
Varnsdorf (Northern Bohemia)	2018, 2019	1	1	6	-	1
Vestec (Central Bohemia)	2020	16	-	-	6	9
Total		24	20	46	25	38

707 [a] MA – *Microtus arvalis*, CG – *Clethrionomys glareolus*, AA – *Apodemus agrarius*, AS –
708 *Apodemus sylvaticus*, AF – *Apodemus flavicollis*.

709

710 Table 5: Prevalence of orthohantavirus RNA in rodents and shrews from the Czech Republic[a].

Species of tested animals	Prevalence (number of positive/number of tested)		
	TULV	**KURV**	**Total**
Microtus arvalis	**70.8% (17/24)**	8.3% (2/24)	79.2% (19/24)
Clethrionomys glareolus	10.0% (2/20)	0% (0/20)	10.0% (2/20)
Apodemus agrarius	10.9% (5/46)	**15.2% (7/46)**	26.1% (12/46)
Apodemus sylvaticus	8.0% (2/25)	8.0% (2/25)	16.0% (4/25)
Apodemus flavicollis	5.3% (2/38)	0% (0/38)	5.3% (2/38)
	SWSV	**ASIV**	
Sorex araneus	**50.0% (2/4)**	**25.0% (1/4)**	75.0% (3/4)
Sorex minutus	0% (0/1)	0% (0/1)	0% (0/1)
Crocidura suaveolens	0% (0/2)	0% (0/2)	0% (0/2)
Crocidura leucodon	0% (0/1)	0% (0/1)	0% (0/1)
Neomys fodiens	0% (0/2)	0% (0/2)	0% (0/2)

711 [a]Viral RNA was detected by nested RT-PCR with universal primer pairs targeting
712 orthohantavirus RNA in all available tissue samples. Orthohantaviruses were identified based
713 on sequencing of a portion of the large (and medium) segment of orthohantavirus genomic
714 RNA. TULV – Tula virus; KURV – Kurkino virus; SWSV – Seewis virus; ASIV – Asikkala
715 virus.
716
717

718 Table 6: Tissue tropism and detection efficiency of orthohantavirus RNA in different tissue
719 samples of the orthohantavirus RNA positive individuals[a].

Virus	Positive individuals	Lungs	Kidneys	Liver	Spleen	Brain	Heart
TULV	28	82.1% (23/28)	52.4% (11/21)	65.2% (15/23)	16.7% (1/6)	0% (0/2)	n.a.
KURV	9	55.6% (5/9)	0% (0/3)	71.4% (5/7)	71.4% (5/7)	75.0% (3/4)	0% (0/2)
SWSV	2	50.0% (1/2)	0% (0/1)	50.0% (1/2)	0% (0/1)	50.0% (1/2)	100% (1/1)
ASIV	1	100% (1/1)	n.a.	n.a.	n.a.	100% (1/1)	100% (1/1)
Total	**40**	**75.0% (30/40)**	**44.0% (11/25)**	**65.6% (21/32)**	**42.9% (6/14)**	**55.6% (5/9)**	**50.0% (2/4)**

720 [a]The percentage was calculated as the ratio of positive samples of the particular tissue to the
721 total number of positive individuals with this tissue sample available (not all tissues were
722 sampled from all individuals). TULV – Tula virus*;* KURV – Kurkino virus*;* SWSV – Seewis
723 virus*;* ASIV – Asikkala virus; n.a. – not available.
724
725

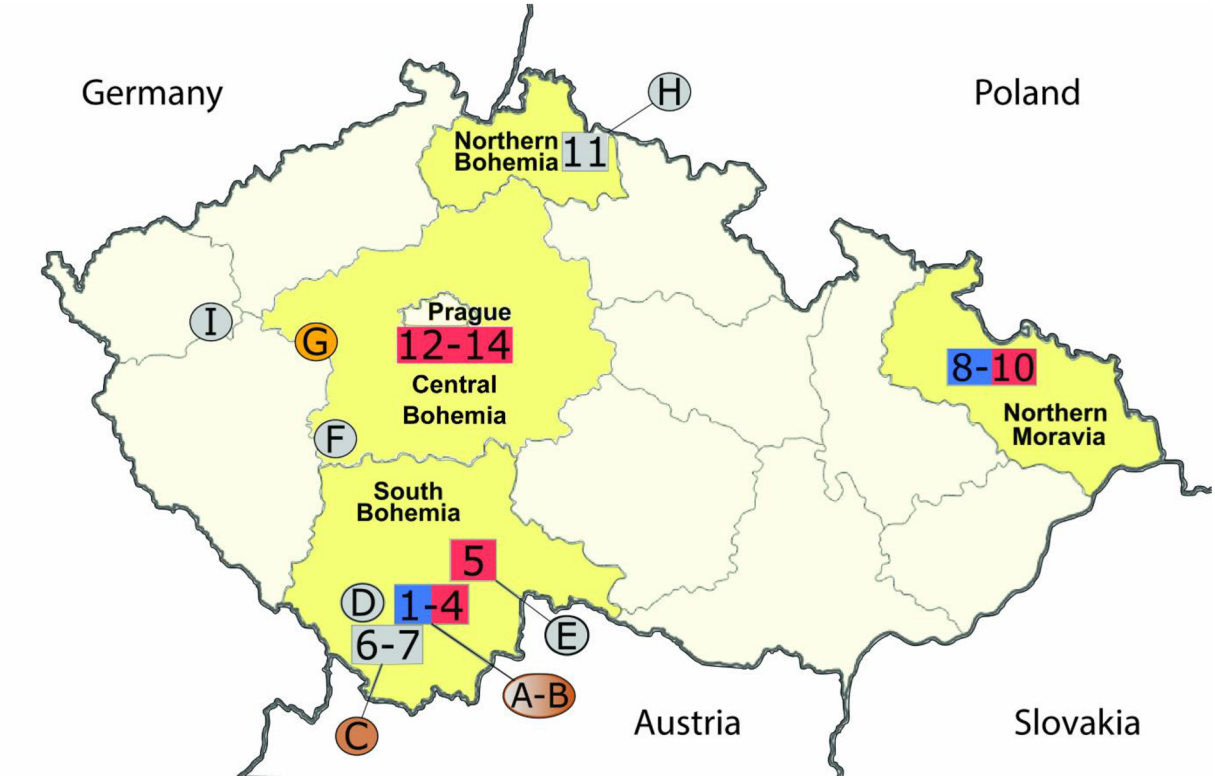

Figure 1. Geographical distribution of the localities used for rodent trapping, and places where the dead shrews were found. Localities of rodent trapping are marked by numbers according to Table 1. Localities of collected shrews are marked by letters as in Table 2. Colour indicates detected orthohantaviruses: red – Tula virus; blue – Kurkino virus; brown – Seewis virus; orange – Asikkala virus; grey - locality where no orthohantavirus RNA-positive samples were detected.

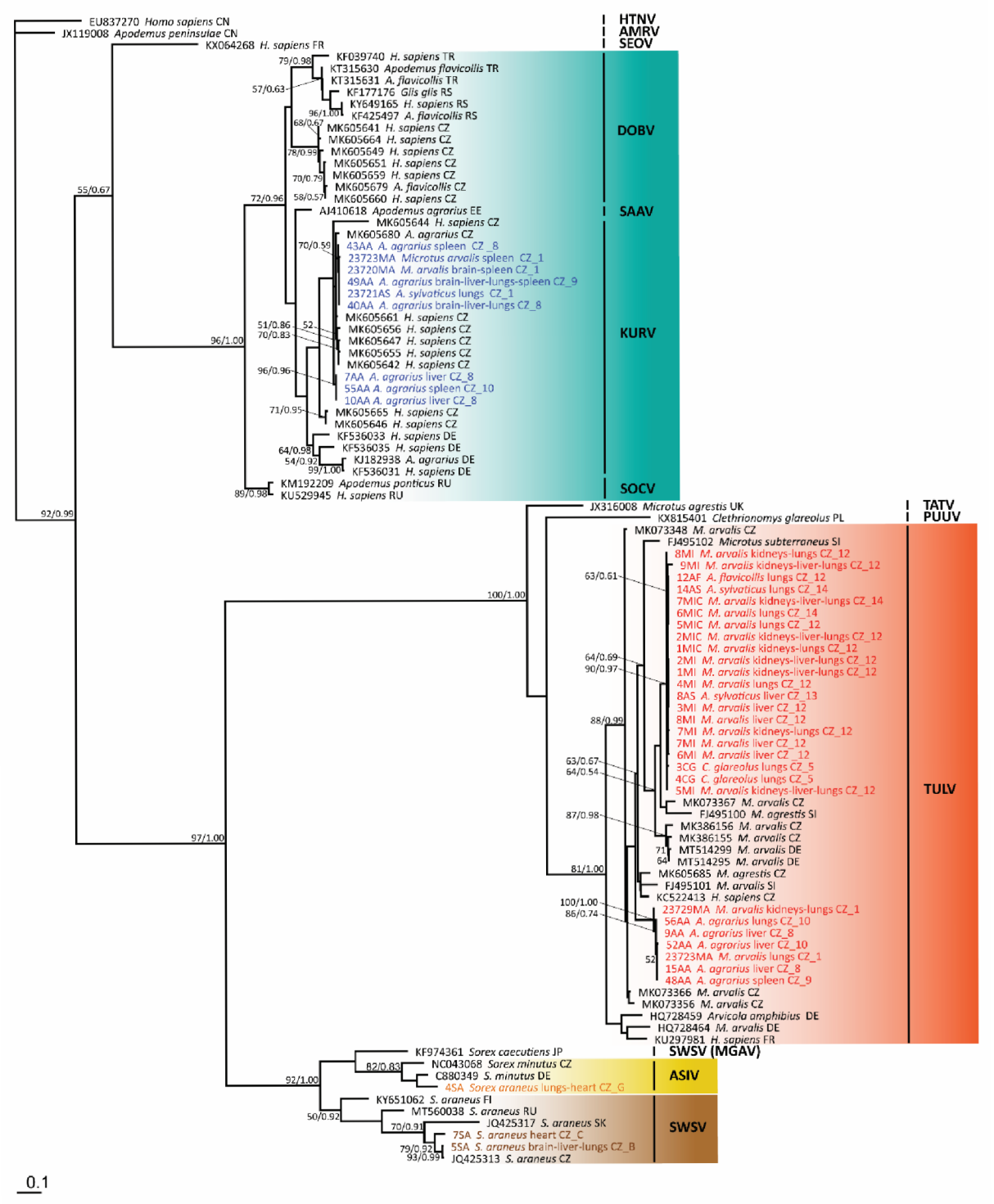

Figure 2. Phylogenetic relationships of the obtained sequences of orthohantaviruses inferred by the maximum likelihood (ML) analysis of the RNA-dependent RNA polymerase gene (L segment). The Bayesian inference (BI) tree was mapped on the ML tree. Numbers at the nodes show bootstrap values derived from the ML analysis/posterior probabilities under the BI analysis. Bootstrap supports and posterior probabilities lower than 50 % or 0.50, respectively, are not provided. Hantaan virus was used as an outgroup. Colours indicate the orthohantavirus: blue = viruses of *Dobrava-Belgrade orthohantavirus* species; red = Tula virus; brown = Seewis

743 virus; yellow = Asikkala virus. Each original sample code consists of the abbreviation of the
744 specific code of the sample, host species, country code, and the map reference (Fig. 1/Table 1).
745 CZ, Czech Republic; DE, Germany; EE, Estonia; FI, Finland; FR, France; JP, Japan; PL,
746 Poland; CN, China; RS, Serbia; RU, Russia; SI, Slovenia; SK, Slovakia; TR, Turkey; UK,
747 United Kingdom.
748

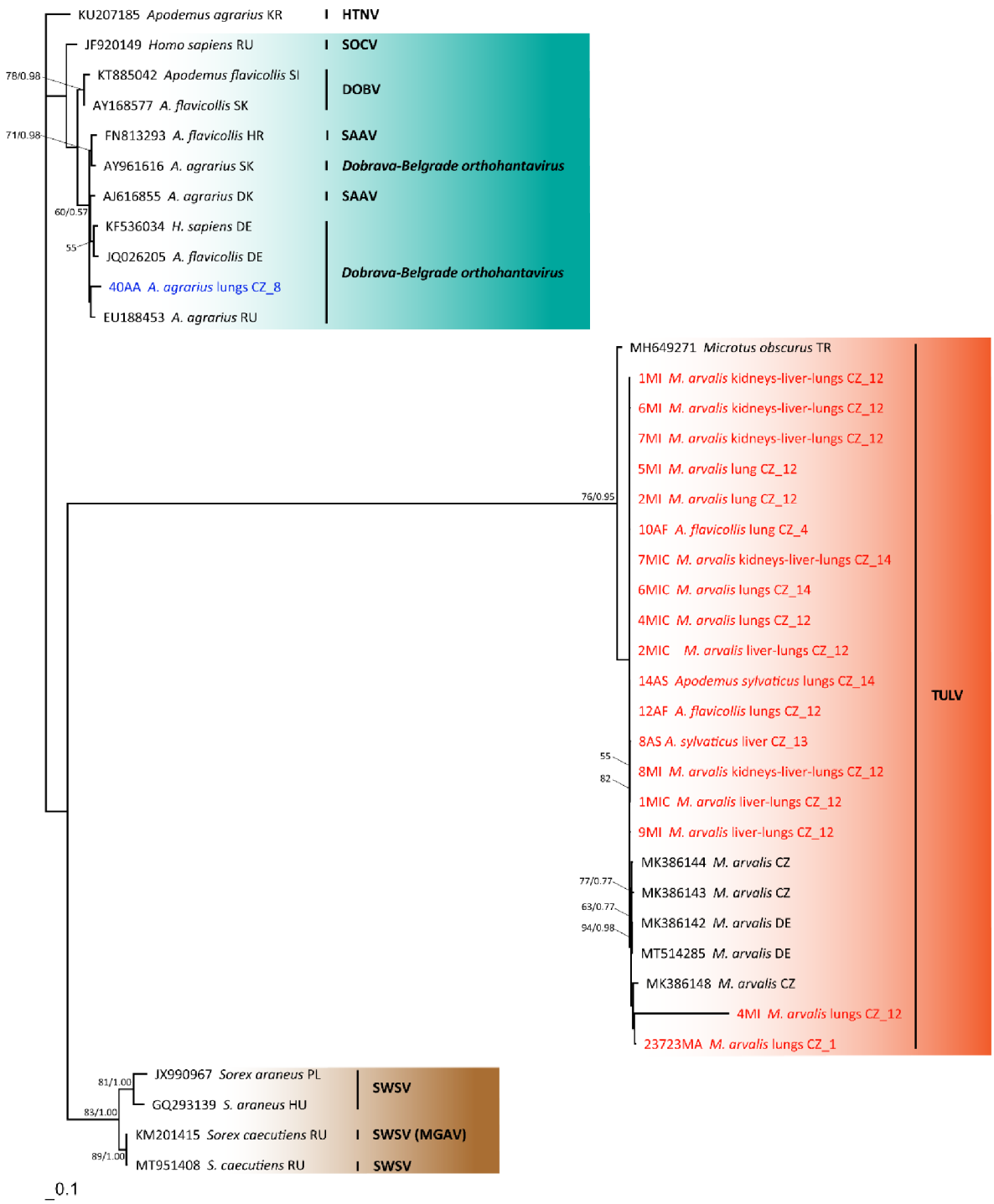

Figure 3. Phylogenetic relationships of the obtained sequences of orthohantaviruses inferred by the maximum likelihood (ML) analysis of the glycoprotein precursor gene (M segment). The Bayesian inference (BI) tree was mapped on the ML tree. Numbers at the nodes show bootstrap values derived from the ML analysis/posterior probabilities under the BI analysis. Bootstrap supports and posterior probabilities lower than 50 % or 0.50, respectively, are not provided. Hantaan virus was used as an outgroup. Colours indicate the orthohantavirus: blue = viruses of *Dobrava-Belgrade orthohantavirus* species; red = Tula virus; brown = Seewis virus. Each

original sample code consists of the abbreviation of the specific code of the sample, species of the host, country code, and the map reference (Fig. 1/Table 1). CZ, Czech Republic; DE, Germany; HR, Croatia; HU, Hungary; KR, South Korea; PL, Poland; SI, Slovenia; SK, Slovakia; RU, Russia; TR, Turkey.