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Fig. 1. Rodent collecting localities in Hungary (shown as full circles). Two localities (TR16 and TR17) that are discussed in the article are marked. Stars indicate localities from where hantavirus sequences have been recovered earlier (Scharninghausen et al., 1999; Jakab et al., 2007a, 2008).
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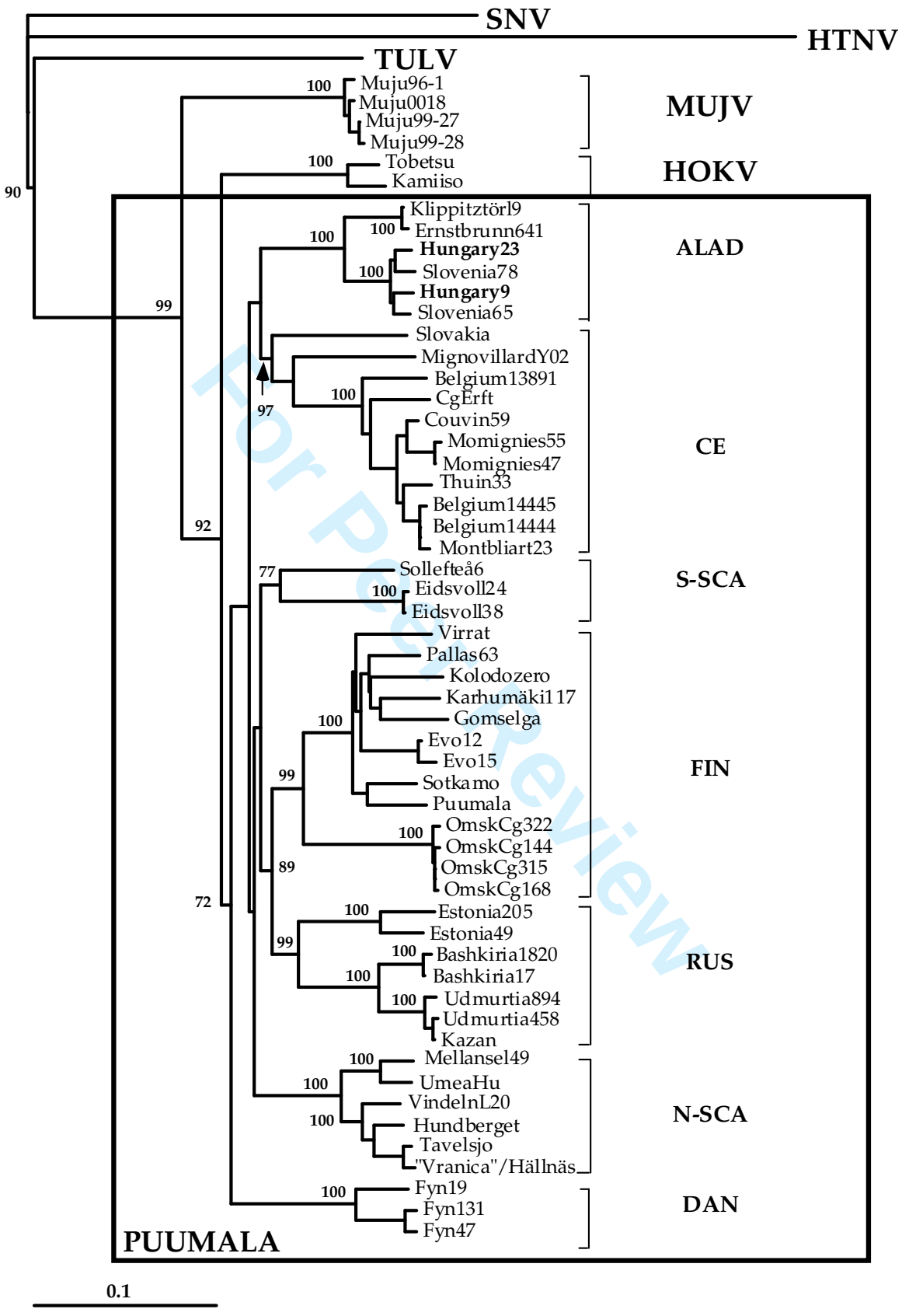

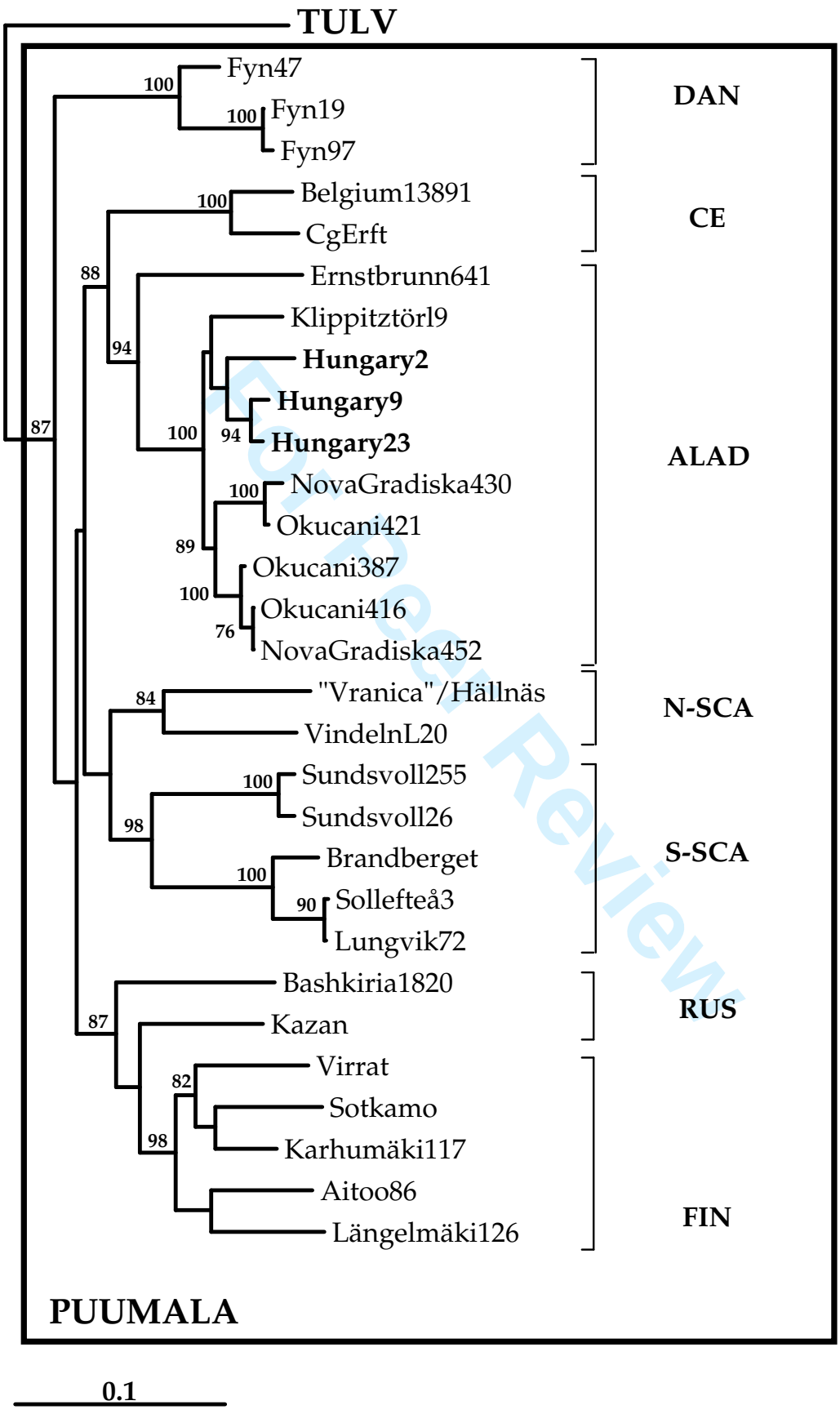

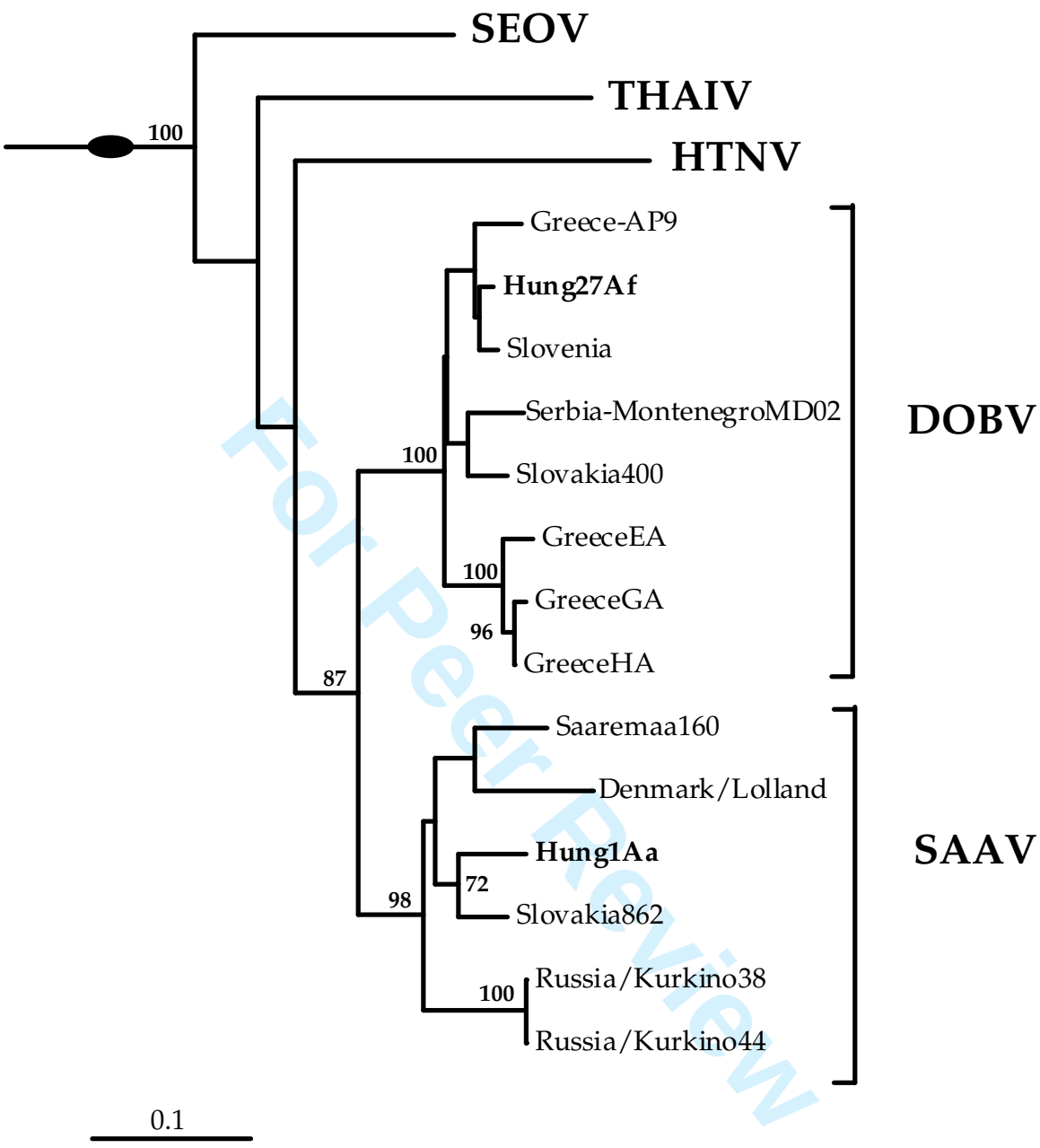

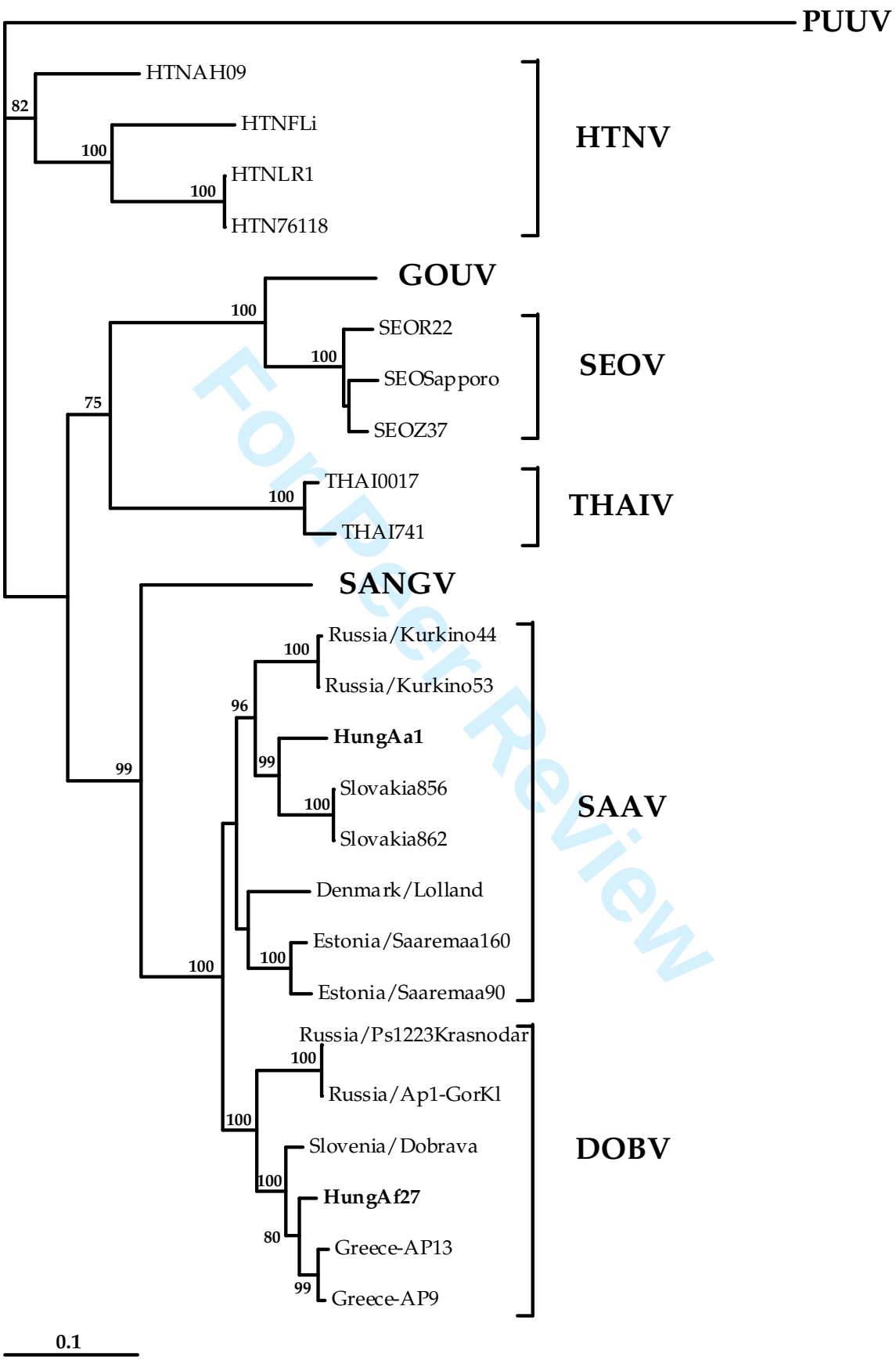

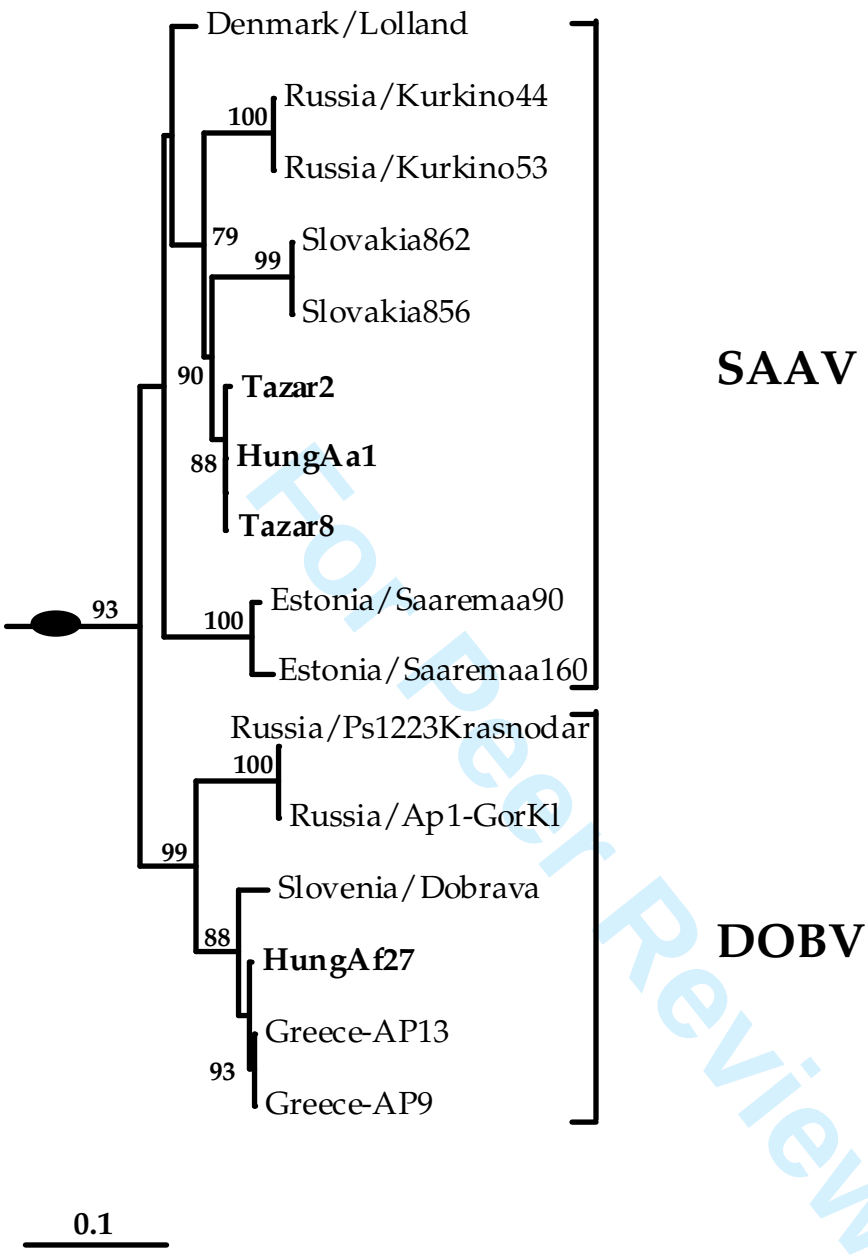

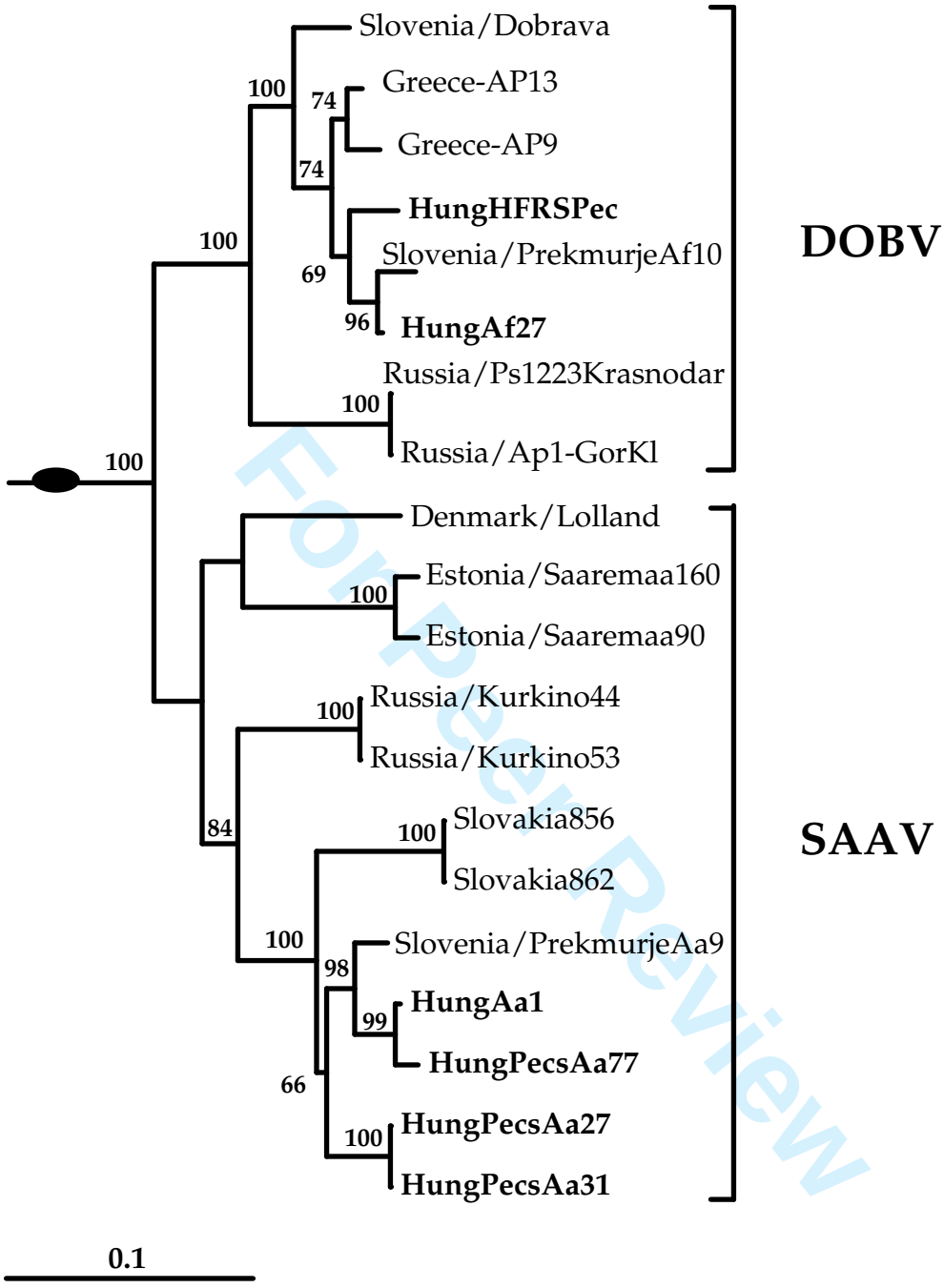

Co-circulation of three pathogenic hantaviruses: Puumala, Dobrava and Saaremaa in Hungary

(Short title: Hantaviruses in Hungary)

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ABSTRACT

Hantaviruses (*Bunyaviridae*) cause hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus (cardio)pulmonary syndrome (HCPS) in the Americas. HFRS is caused by Hantaan (HTNV), Seoul (SEOV), Dobrava (DOBV), Saaremaa (SAAV) and Puumala (PUUV) viruses. Of those, only HTNV is not present in Europe. In recent years, hantaviruses, described in other parts of Europe, were also detected at various locations in Hungary. To study the genetic properties of Hungarian hantaviruses in detail, sequences of the viral S and M segments were recovered from bank voles *(Myodes glareolus*), yellow-necked mice (*Apodemus flavicollis*) and striped field mice (*Apodemus agrarius*) trapped in the Transdanubian region. As expected, the sequences recovered belonged, respectively, to PUUV (two strains), DOBV (one strain) and SAAV (one strain). On phylogenetic trees two new Hungarian PUUV strains located within the well-supported Alpe-Adrian (ALAD) genetic lineage that included also Austrian, Slovenian and Croatian strains. Analysis of the Hungarian SAAV and DOBV genetic variants showed host-specific clustering and also geographical clustering within each of these hantavirus species. Hungarian SAAV and DOBV strains were related most closely to strains from Slovenia (Prekmurje region). This study confirms that multiple hantaviruses can co-circulate in the same locality and can be maintained side-by-side in different rodent species.

Key words: HFRS; hantavirus; Puumala virus, Dobrava virus, Saaremaa virus.

INTRODUCTION

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) cause hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus (cardio)pulmonary syndrome (HCPS) in the Americas (for reviews, see Schmaljohn and Hjelle, 1997; Vapalahti et al., 2003). HFRS is caused by *Hantaan virus* (HTNV), *Seoul virus* (SEOV), *Dobrava-Belgrade virus* (DOBV), *Saaremaa virus* (SAAV) and *Puumala virus* (PUUV). *Sin Nombre virus* (SNV), *Andes virus* and related hantaviruses are the main causative agents of severe HCPS. Hantaviruses are host-specific, and thus their distribution correlates most often with the geographical distribution of the natural host species, rodents and insectivores. Transmission of hantaviruses to humans occurs by inhalation of infested aerosols of rodent excreta. Whether hantaviruses carried by insectivores can infect humans and cause any disease remains to be seen.

Of HFRS-causing hantaviruses, only HTNV is not present in Europe [Vapalahti et al., 2003]. PUUV, carried by bank voles *(Myodes glareolus)*, is the most widely distributed European hantavirus. It causes a mild form of HFRS (also called *nephropathia epidemica*, NE) in Northern and Central Europe, European part of Russia and in the Alpe-Adrian region. DOBV, harbored by yellow-necked field mice (*Apodemus flavicollis*), is the most severe European hantavirus pathogen; it is associated with HFRS mostly in the Balkans [Papa et al., 1998; Avsic-Zupanc et al., 1999]. SAAV, carried by striped field mice (*Apodemus agrarius*), causes mild HFRS resembling NE; the virus is found in the Baltics, Central Europe and European part of Russia [Lundkvist et al., 1998; Plyusnin et al., 1999; Golovljova et al., 2000; Sibold et al., 2001]. SEOV, carried by Norway rats (*Rattus norvegicus*), is so far found in

France and Belgium [Heyman et al., 2004, 2009]. Apart from laboratory outbreaks caused by SEOV-infected rats kept in captivity, no human cases in Europe have been reported in connection to this hantavirus.

HFRS has been described in Hungary prior to the identification of the causative agents, the hantaviruses [Trencsenyi and Keleti, 1981]. Until 1985, human HFRS cases were diagnosed initially based on medical symptoms and epidemiology; these were later confirmed using serological tests [Takenaka et al., 1985; Faludi and Ferenczi 1995]. With the advent of DNA and RNA sequencing several hantaviruses have been identified in Hungary [Scharninghausen, 1999, Ferenczi 2003, Jakab 2007a, 2008].

On average, there are 20-25 laboratory-confirmed human hantavirus infections in Hungary each year. The fatality rate associated with clinical infections dropped from approximately 6% in the 1950s to 2.5% in recent years. In case a physician suspects HFRS, further serological tests are performed at the National Center for Epidemiology (NCE). Serological diagnosis is based primarily on immunofluorescence assay (IFA) and secondarily on enzyme-immunoassay (EIA). Compared to the number of hospitalized patients, hantavirus seroprevalence is much higher in Hungary, approximately 10% [Ferenczi et al., 2005]. These controversial data might be explained by the low HFRS awareness of the medical community and by the undiagnosed mild forms of the disease.

The aim of this study was to recover hantavirus sequences from tissue samples of rodents known as hatural hosts for Puumala, Dobrava and Saaremaaa viruses, perform their detailed (phylo)genetic characterization, assign them to proper taxa and specify unequivocally the rodent hosts involved.

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MATERIALS AND METHODS

Trapping of rodents. Small mammals were collected as part of a larger research project on studying the distribution of hantaviruses in Hungary. The broader area, the southwestern part of Hungary, was selected based on the high incidence rate of human hantavirus cases (Fig. 1). Zala county with only 3% of Hungary's total population gives 23% of HFRS cases in the country. In 2000, selected trapping localities were chosen to survey wild mammals as reservoirs of hantaviruses in forest habitats. The primary factor in selecting specific sites was the forest area, but several other characteristics of the plant community (e.g. disturbance level and plant species composition) correlated with patch size. Rodents were trapped using Sherman type live capture traps [Mills et al., 1995] set in the evening and checked in the morning. At each locality trapping was continued until sufficient numbers of animals (usually around 40-50) were captured. Animals were identified, aged, sexed and their conditions were recorded. Euthanized rodents were dissected; blood and tissue samples – lung, spleen, kidney, heart – were removed for further analysis. Tissues were frozen immediately in liquid nitrogen. Upon arrival at the Virology Department of the NCE tissue samples were transferred to -80°C freezers for long-term storage. Skeletal materials of the collected animals were deposited as voucher specimens at the Mammal Collection of the Museum of Southwestern Biology, University of New Mexico.

Screening of rodent samples. Sera collected from rodents were screened initially for the presence of antibodies to hantaviruses by commercially available kits as described previously [Ferenczi et al., 2003]. Briefly, HTNV antigen-coated

high density particle agglutination (HDPA) kit (Korean Green Cross) was used according the protocol of the WHO Collaborating Center for Virus Reference and Research (HFRS), Institute for Viral Diseases, Korea University, Seoul, South Korea. To perform indirect immunofluorescent assay (IFA), HTNV-, PUUV- and SEOV-specific tests (Progen Biotechnik, GMBH, Germany) with FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO, A/S, Denmark) were used. For the detection of HTNV- and PUUV-specific antibodies by enzyme immunoassay (EIA), IgG and IgM kits (Progen Biotechnik) were applied.

Twenty five out of 37 serum samples were tested additionally on EIA plates containing recombinant antigens for PUUV, DOBV and SEOV viruses [Elgh et al., 1997]. Lung tissue samples from seropositive rodents were screened further for hantavirus nucleocapsid (N) protein antigen by immunoblotting as described earlier [Plyusnin et al., 1995].

Reverse transcription - polymerase chain reaction (RT-PCR) and sequencing. RNA was extracted from lung tissue samples using the Tripure reagent (Boehringer Mannheim) following recommendations of the manufacturer. RNA was then subjected to RT-PCR to recover partial S segment and M segment sequences (sequences of primers and other experimental details are available upon request). PCR amplicons were gel-purified with QIAquick Gel Extraction -kit (QIAGEN) and sequenced directly using ABI PRISM^{TM} Dye Terminator sequencing kit (Perkin Elmer/ABI, NJ). PUUV genome sequences described in this paper have been deposited to the GenBank sequences database under accession numbers FN377821-25. DOBV and SAAV genome sequences described in this paper have been deposited to the GenBank sequences database under accession numbers FN377826-29.

Mitochondrial DNA (mtDNA) analysis was performed as described earlier [Nemirov et al., 2002]. Briefly, DNA was extracted from lung tissue samples using the Tripure reagent. A 427 nt-long PCR-product from the D-loop-encoding region was amplified with primers 5'-CCACCATCAGCACCCAAAGCTG-3' and 5'-CTGAAGTAAGAACCAGATGTCTG-3'. The product was purified from the gel and subjected to direct sequencing. For comparison, mtDNA sequences of *A. agrarius* and *A. flavicollis* were retrieved from the GenBank nucleotide databases.

Phylogenetic analysis. Multiple nucleotide alignments were prepared manually using SeqApp 1.9a169 sequence editing program. Phylogenetic analysis was performed using the PHYLIP program package [Felsenstein, 1993]. 500 bootstrap replicates (Seqboot program) were submitted to the distance matrice algorithm (Dnadist program), with maximum likelihood model for nucleotide substitutions); distance matrices were analysed with the Fitch-Margoliash tree-fitting algorithm (Fitch program); the bootstrap support values were calculated with the Consense program. Hantavirus sequences used for comparison were recovered from the GenBank.

RESULTS

Prescreening of rodents. Rodent candidates for further RT-PCR and sequencing were pre-screened for hantavirus antibodies by HDPA, IFA and EIA. From more than 300 rodents, 37 were found positive or suspected positive: 8 bank voles (*Myodes glareolus*) and 29 *Apodemus* mice (18 *A. agrarius*, 2 *A. flavicollis*, 9 - unspecified *Apodemus* spp.). Positive *M. glareolus* were from the locality TR17, positive *Apodemus* mice from localities TR16 and TR17 (Fig. 1).

Genetic analysis of PUUV strains. From eight pre-selected (Ab-positive) bank voles, six were found positive for PUUV N-Ag in immunoblotting. Of those, three appeared also positive for PUUV genome when tested by RT-PCR; all three animals were from the same locality, TR-17. Three wild-type PUUV strains (that were not isolated) were designated PUUV/Mg2/HungaryTR17/00, PUUV/Mg9/HungaryTR17/00, and PUUV/Mg23/HungaryTR17/00, or Hung2, Hung9, and Hung23, for short. For two strains, Hung9 and Hung23, almost complete sequences of the S segment have been recovered in a set of (semi)-nested PCRs. For the strain Hung2, only partial sequence of the coding region (nt 191 to 1190) was recovered. It differed by two silent nucleotide substitutions from the corresponding sequence of strain Hung9 and was excluded from further analyses. Two other sequences included 5'-noncoding region (NCR) of 42 nt and the open reading frame (ORF) for the N protein of 433 aa followed by 499 or 442 nt from 3'-NCR (for strains Hung9 and Hung23, respectively). The 5'-NCR sequences were identical (the first 22 nt originated from the primer sequence and thus were not determined directly); the coding regions differed by 34 substitutions (identity of

97.4%); the 3'-NCR sequences differed at 28 positions (identity of 95.9%). Deduced N protein sequences differed by one substitution only (identity of 99.8%) and carried signature residues V236 and P257 shared also by PUUV strains from neighbouring Austria [Plyusnina et al., 2006] and Slovenia [Avsic-Zupanc et al., 2007]. Partial M segment sequences (nt 2161-2570, primers excluded) of strains Hung2, Hung9, and Hung23 were recovered as well; they appeared 94.4-96.1% identical to each other. The encoded partial sequences of the Gc protein (136 aa long) were 97.8-99.3% identical. Hungarian M segment/Gc protein sequences were most closely related to the corresponding sequences from Austrian [Plyusnina et al., 2006] and Croatian [Cvetko et al., 2005] PUUV strains (corresponding sequences from Slovenian strains are not available).

On the phylogenetic tree based on the complete S segment coding region, the two Hungarian PUUV strains were located within the well-supported Alpe-Adrian (ALAD) genetic lineage that included also Austrian and Slovenian strains (Fig. 2A). Within this lineage, Hungarian and Slovenian strains shared the most recent common ancestor (TMRCA) while Austrian strains formed a sister taxon to this quartet. Six other PUUV lineages can be recognized: (1) Central European (CE); (2) South Scandinavian (S-SCA); (3) Finnish (FIN); (4) Russian (RUS); (5) North Scandinavian (N-SCA); and (6) Danish (DAN). PUUV shared more ancient common ancestors (MACAs) with Hokkaido virus (HOKV) and then with Muju virus (MUJV) associated with *Myodes rufocanus* and *Myodes regulus,* respectively. The phylogenetic tree based on the partial M segment sequence showed similar topology (Fig. 2B): Hungarian strains were located within the ALAD lineage which, in this

case, included also strains from Austria and Croatia. Other PUUV lineages (CE, S-SCA, FIN, RUS, N-SCA and DAN) were presented as well.

Genetic analysis of SAAV and DOBV strains. From 29 pre-screened *Apodemus* mice (18 *A. agrarius,* 2 *A. flavicollis,* 9 - unspecified); six were found positive for DOBV/SAAV N-Ag in immunoblotting. Of those, only two were positive also in the RT-PCR test. Analysis of mtDNA sequences showed that one mouse (#1, from locality TR-16) belonged to the species *A. agrarius,* another (#27 from locality TR-17) was *A. flavicollis.* Partial S and M segment sequences were recovered from both rodents using DOBV/SAAV-specific primers and subjected to phylogenetic analysis. On phylogenetic trees (Fig. 3A-B), the sequence recovered from *A. agrarius* mouse was placed with SAAV sequences (all recovered from striped field mice) while the sequence recovered from *A. flavicollis* mouse co-localized with DOBV sequences, originated from either yellow-necked mice or human HFRS cases. Although the topologies of the trees based on partial M and S segment sequences were quite similar, the M-tree showed a somewhat better resolution, i.e. higher bootstrap support values for SAAV- and DOBV-clades. Based on these data, novel Hungarian hantavirus strains were designated SAAV/Aa1/Hungary TR16/00 and DOBV/Af27/HungaryTR17/00 or HungAa1 and HungAf27, for short.

New Hungarian SAAV and DOBV sequences were compared to previously published ones recovered from *A. agrarius* trapped in Taszár [spelled as "Tazar" in the original publication], eastern Hungary [Scharninghausen et al., 1999]. The analysis was restricted to the overlapping part of the S segment sequence recovered in two studies: nt 707 to 935. This region of two Taszár strains appeared most

closely related to the corresponding region of the SAAV strain HungAa1: only one (silent) nucleotide substitution was observed between these sequences (identity of 99,6%). In contrast, the sequence of the DOBV strain HungAf27 differed from Taszár sequences at 29-30 positions (identity of 86.9-87.3%). Phylogenetic analysis showed TMRCA for the two Taszár strains and the strain HungAa1 (Fig. 3C). This trio was placed within the SAAV clade and shared MACA with SAAV strains from Slovakia. These data suggested that the two Taszár strains belong to SAAV.

New Hungarian SAAV and DOBV sequences were compared also to the previously published sequences recovered from *A. agrarius* trapped in the Görcsöny and Sármellék areas and from an HFRS patient from the University hospital of Pécs (all in the Transdanubian region) [Jakab et al., 2007a,b]. Since, in this case, the analysis was restricted to shorter sequences of the S segment recovered by these authors (nt 410 to 800), it became possible to include also partial SAAV and DOBV sequences originated from Prekmurje region of neighbouring Slovenia [Avsic-Zupanc et al., 2000].

Direct sequence comparison showed that Hungarian SAAV strain HungAa1 was most closely related to Hungarian strain Pecs/77Aa from Sármellék (sequence identity 99.5%) and to Slovenian SAAV strain Aa9 from Prekmurje (97.9%). Sequence of our DOBV strain HungAf27 was most closely related to the corresponding region of Slovenian DOBV strain from Prekmurje (99.2%) and then to Hungarian HFRS-associated strain (92.0%). On the phylogenetic tree (Fig. 3D) all four Hungarian *A. agrarius* originated sequences, as expected, were located within the SAAV clade. The new strain HungAa1 shared TMRCA with the strain PecsAa77 and this couple shared the MACA with Slovenian strain Aa9 from Prekmurje.

Similarly, our DOBV strain HungAf27 shared TMRCA with Slovenian DOBV strain Af10 from Prekmurje; they, in turn, shared MACA with the Hungarian HFRS-associated strain. These results demonstrated host-specific clustering of Hungarian SAAV and DOBV genetic variants and, within each of these hantavirus species, also geographical clustering.

DISCUSSION

The results of this study demonstrate co-circulation of all three major European hantavirus pathogens in Hungary: PUUV, DOBV and SAAV. They are in line with earlier observations on human HFRS cases in this country [Ferenczi et al., 2005; Jakab et al., 2007a] and recovery of hantavirus sequences from rodents [Scharninghausen et al., 1999; Ferenczi et al., 2003; Jakab et al., 2007a]. It should be emphasized that while SAAV in Hungarian striped field mice has been already reported (albeit under the name of Dobrava virus - see below), the first unequivocal evidence for PUUV and DOBV in Hungarian rodents, species *M. glareolus* and *A. flavicollis*, respectively, is presented in this paper.

Rodents used in genetic studies were selected in two steps. First, pre-selection by HDPA, EIA and IFA to detect hantavirus antibodies was performed. On that stage, not only clearly Ab-positive but also borderline voles and mice were selected for further analyses. This could explain the observed differences between the results of antibody detection and the N-Ag detection, due to pre-selection not only clearly Ab-positive but also voles and mice giving the borderline results. In turn, the relatively low RT-PCR positivity of N-Ag-positive rodents could be due to partial degradation of target RNA in tissue samples that were subjected to several thawing-freezing cycles (to take material for other analyses) before RNA extraction.

Phylogenetic analysis of Hungarian PUUV strains placed them within the ALAD genetic lineage that includes also strains from neighbouring Slovenia, Croatia and Austria. This presents a good example of geographical clustering of PUUV genetic variants, a phenomenon first discovered more than a decade ago [Plyusnin

et al., 1994]. Phylogenies that included new Hungarian PUUV sequences confirmed two interesting earlier observations [Plyusnina et al., 2006]. First, within the ALAD lineage, two Austrian M-sequences are not monophyletic. One of the strains, Klipp9, is clustering together with Croatian and Hungarian strains suggesting that M and S segments of this strain might have different evolution history, i.e. this strain is a reassortant one. Second, ALAD and CE lineages and RUS and FIN lineages share MACAs thus suggesting somewhat closer relationships within these two pairs of lineages that could be rooted to the last postglacial recolonization of these areas of Europe.

The analyses of Hungarian SAAV and DOBV sequences confirmed that genetic variants of these hantavirus species show the host-specific clustering. Indeed, all sequences recovered from *A. agrarius* belonged to SAAV while those from *A. flavicollis* belonged to DOBV. Thus previously reported sequences from Hungarian *A. agrarius* [Scharninghausen et al., 1999; Jakab et al., 2007a] should be re-classified as SAAV strains. It seems that only the sequence recovered from human HFRS case in Pécs [Jakab et al., 2007b] presents *bona fide* DOBV. As mentioned above, for several years there was a controversy in taxonomy and hence terminology of SAAV. When first discovered, this Dobrava-like virus was erroneously considered a genetic variant of DOBV carried by the striped field mice [Plyusnin et al., 1997; Nemirov et al., 1999]. It took several years to realize that this is in fact a distinct hantavirus species [Brus-Sjölander et al., 2002; Nemirov et al., 2002], the view that is now shared by the International Committtee for Virus Taxonomy [www.ictvonline.org].

Another important finding of this study is that multiple hantavirus types can be maintained at the same locality by their corresponding rodent hosts. The direct

transmission mode and the high level of host specificity of the hantaviruses make these temperate-zone viruses less affected by host biodiversity [Ruedas et al., 2004]. Instead, the population density and size are the main factors driving the disease temporal dynamics.

Of the four European hantavirus pathogens known so far, only SEOV has not been found in Hungary, albeit serological evidence showed its causative role at least in one case [Ferenczi, unpublished data]. Direct detection of this virus is rather difficult because its primary hosts, Norway rats *Rattus norvegicus*, would not be caught by the traps used. Similar observation have been made in neighbouring Slovenia [Avsic-Zupanc et al., 2002, 2007], Slovakia [Sibold et al., 2001] and Croatia [Cvetko et al., 2004]. Our unpublished serological data on clinical cases confirm that both PUUV-like and DOBV/SAAV-like HFRS cases occur in Hungary. Co-circulation of three distinct hantaviruses that could cause HFRS of different severity, including life-threatening infections, calls for improvements in both surveillance and diagnostics.

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Legends to figures

Fig. 1. Rodent collecting localities in Hungary (shown as full circles). Two localities (TR16 and TR17) that are discussed in the article are marked. Stars indicate localities from where hantavirus sequences have been recovered earlier (Scharninghausen et al., 1999; Jakab et al., 2007a, 2008).

Fig. 2. Phylogenetic trees of Puumala and related hantaviruses based on **(A)** complete S segment coding sequences and **(B)** partial M segment sequences (nt 2161-2570). Numbers show the bootstrap support values for the branching points (500 replicates). Only the values higher than 70% are shown. Abbreviations: **SNV,** Sin Nombre virus, strain NM H10; **HTNV**, Hantaan virus, strain 76-118; **TULV**, Tula virus, strain Moravia02v; **MUJV**, Muju virus; HOKV, Hokkaido virus. Genetic lineages of PUUV: **ALAD**, Alpe-Adrian; **CE**, Central European; **S-SCA**, South Scandinavian; **FIN**, Finnish; **RUS**, Russian; **N-SCA**, North Scandinavian; **DAN**, Dannish.

Fig. 3. Phylogenetic trees of Dobrava, Saaremaa and related hantaviruses based on **(A)** partial M segment sequences (nt 1704-1969), and partial S segment sequences: **(B)** nt 377-935; **(C)** nt 707-935; **(D)** nt 410-800. Numbers show the bootstrap support values for the branching points (500 replicates). Only the values higher than 50% are shown. Abbreviations: **DOBV,** Dobrava virus; **SAAV,** Saaremaa virus; **HTNV**, Hantaan virus, strain 76-118 (if not specified otherwise); **SEOV**, Seoul virus, strain Sapporo rat (if not specified otherwise); **THAIV**, Thailand virus, strain741; **GOUV**, Gou virus, strain Nc167; **SANGV**, Sangassou virus strain SA14; **PUUV,** Puumala virus, strain Sotkamo.