

## COMPARATIVE PHYLOGENETIC ANALYSIS OF DOBRAVA-BELGRADE VIRUS L AND S GENETIC SEGMENTS ISOLATED FROM AN ANIMAL RESERVOIR IN SERBIA

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**Abstract** – The Dobrava-Belgrade virus (DOBV) is a member of the *Bunyaviridae* family, genus *Hantavirus*, possessing a single-stranded RNA genome consisting of three segments, designated L (large), M (medium) and S (small). In this study, we present phylogenetic analysis of a newly detected DOBV strain isolated from *Apodemus agrarius*. Analysis was based on partial L and S segment sequences, in comparison to previously published DOBV sequences from Serbia and elsewhere. A phylogenetic tree based on partial S segment revealed local geographical clustering of DOBV sequences from Serbia, unrelated to host (rodent or human). The topology of the phylogenetic tree was confirmed with a high percent of completely or partially resolved quartets in likelihood-mapping analysis, whereas no evidence of possible recombination in the examined S segment data set was found.

**Key words:** DOBV; phylogenetic analysis; likelihood mapping; recombination

### INTRODUCTION

Hantaviruses are the causative agents of two human zoonotic disease: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) with mortality rates of up to 12%-18% (HFRS) and 60%, respectively (Kovacevic et al., 2008; Papa, 2012). These are enveloped viruses, members of the family *Bunyaviridae*, with a negative sense RNA genome consisting of three segments, large (L) (6.5-6.6kb), medium (M) (3.6-3.7kb) and small (S) (1.7-2.4kb), which encode a RNA-dependent RNA polymerase, the G1 and G2 envelope glycoproteins

and the nucleocapsid protein, respectively (Elliott et al., 1991). In contrast to other members of the family *Bunyaviridae*, hantaviruses do not have an arthropod vector. These agents are primarily rodent-borne, having coevolved with their natural hosts, members of two different rodents families, *Muridae* (subfamily *Murinae*) and *Cricetidae* (subfamilies *Arvicolinae*, *Neotominae* and *Sigmodontinae*) (Kang et al., 2011). Recent discovery of genetically new shrew- and mole-associated hantaviruses indicates that the evolutionary history of hantaviruses is very complex (Kang et al., 2011). Additionally, the results of new studies confirmed the presence of hantaviruses in

*Nycteris hispida*, a bat originating from Sierra Leone, West Africa (Weiss et al., 2012). It is known that each hantavirus is predominantly associated with one rodent species. Thus, *Apodemus flavicollis* is associated with Dobrava-Belgrade (DOBV) virus, *Myodes glareolus* with Puumala (PUUV), *Rattus norvegicus* with Seoul (SEOV), and *Microtus arvalis* with Tula virus (TULV). Nevertheless, multiple studies revealed that single hantaviruses can be detected in different rodent reservoirs, e.g. DOBV was also found in *Apodemus agrarius* and *Apodemus ponticus* (Schlegel et al., 2009).

The distribution of over 20 different hantaviruses, causative agents of human illnesses, depends on the geographic distribution of their natural reservoirs (Jonsson et al., 2010). Five different old-world hantaviruses are known to be present in Europe, including Puumala (PUUV), Hantaan (HTNV), Dobrava-Belgrade (DOBV), Seoul (SEOV) and Tula virus (TULV) (Heyman et al., 2011). All these hantaviruses, excluding TULV, were proven to be causative agents of HFRS (Vapalahti et al., 2003). Several human cases of TULV infection have been documented, but there is no strong evidence of association between this virus and infections (Schultze et al., 2002; Clement et al., 2003; Klempa et al., 2003a). Direct contact with infected rodents or with their aerosolized excreta are proposed to be the primary root of human infection (Mir, 2010).

DOBV was described as a human isolate for the first time in Serbia in 1992 (Gligic et al., 1992). Soon afterwards, analysis of a virus genome isolated from *A. flavicollis* captured in Slovenia confirmed the existence of new hantavirus strain (Avsic-Zupanc et al., 1992). Based on the limited comparison of DNA sequences, these two isolates are considered to be one virus – Dobrava-Belgrade (Taller et al., 1993, Xiao et al., 1993).

Four different lineages of DOBV, hosted by different *Apodemus* species, circulate in Europe and Russia. DOBV-*Af* lineage is associated with yellow-necked mouse (*A. flavicollis*), DOBV-*Aa* and Saaremaa lineages are associated with striped field mouse

(*A. agrarius*), and DOBV-*Aplineage* is associated with Caucasian wood mouse (*A. ponticus*) (Papa, 2012). A newly proposed classification allocates DOBV in four genotypes corresponding to the four above-listed lineages: Dobrava genotype analogous to DOBV-*Aplineage*; Kurkino genotype, named for the location of the first DOBV-*Aa* lineage isolation; Sochi genotype, corresponding to the DOBV-*Ap* lineage; and finally, Saaremaa genotype, also carried by *A. agrarius* (Klempa et al., 2013).

The aim of the present study was to characterize the newly detected hantavirus sequence from Serbia and to describe the phylogenetic relatedness of this sequence to previously published ones, using different bioinformatic tools.

## MATERIALS AND METHODS

### *RT-PCR and sequencing*

Phylogenetic analysis included partial L and S segments of the newly detected DOBV and all sequences matching the analyzed regions taken from the GenBank (<http://www.ncbi.nlm.nih.gov>). Total RNA of *Apodemus agrarius* lung tissue was extracted using TRIZOL Reagent (GibcoBRL, Invitrogen, Karlsruhe, Germany). New sequences of partial L and S segments of DOBV were obtained by nested-PCR protocols using a One-Step RNA PCR Kit (Qiagen, Hilden, Germany) for the outer reaction and a Taq PCR Core Kit (Qiagen, Hilden, Germany) for inner PCR reaction. Partial L segment amplification was performed with primers compatible to all known hantaviruses (Table 1) (Klempa et al., 2006). In order to detect a significant part of the DOBV S segment-encoding region, we designed primers finally amplifying 1323 nt inner product (Table 1), using the appropriate tool and based on existing DOBV S segment sequences in the database (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The designed primers afforded amplification of an almost complete coding region of the DOBV S segment (Table 1).

The PCR protocol for partial S segment amplification included an initial step for 10 min at 65°C.

The purpose of this procedure was relaxation of RNA molecules' secondary structures. Both PCR products were sequenced by dye-terminator sequencing on an ABI 310 automated DNA sequencer (Applied Biosystem, Foster City, CA, USA).

#### *Sequence data sets*

Detailed phylogenetic analysis was conducted using data sets of six L segment and 66 S segment sequences retrieved from GenBank. The accession numbers of the analyzed L segment sequences are NC005235, JQ026206, JF920148, GU904039, GU904042, and AJ410618. The accession numbers of the analyzed S segment sequences are NC005233, JQ026204, JF920150 to JF920152, AY168576, FN813291, GU904027 to GU904032, EU562989 to EU562991, AF442622, AF442623, GQ205401 to GQ205408, AY533120, AY961615, AY533118, AY961618, EU188449, EU188452, AJ616854, AJ410619, AJ410615, AJ131672, AJ131673, AJ269549, AJ009775, AJ269550, AJ009773, JQ344114, JF499666, HQ174468 to HQ174470, FN813292, FJ986109, GQ205393 to GQ205398, AF0600214 to AF0600224, FN377828, FN377826, DQ305279 to DQ305281, AJ269554, AJ251996 and AJ251997.

Phylogenetic trees were rooted using different hantavirus reference sequences as outgroups: HTNV (NC005218 for S segment and NC005238 for L segment) and SEOV (NC005236 for S segment and NC005222L for L segment).

#### *Phylogenetic analysis*

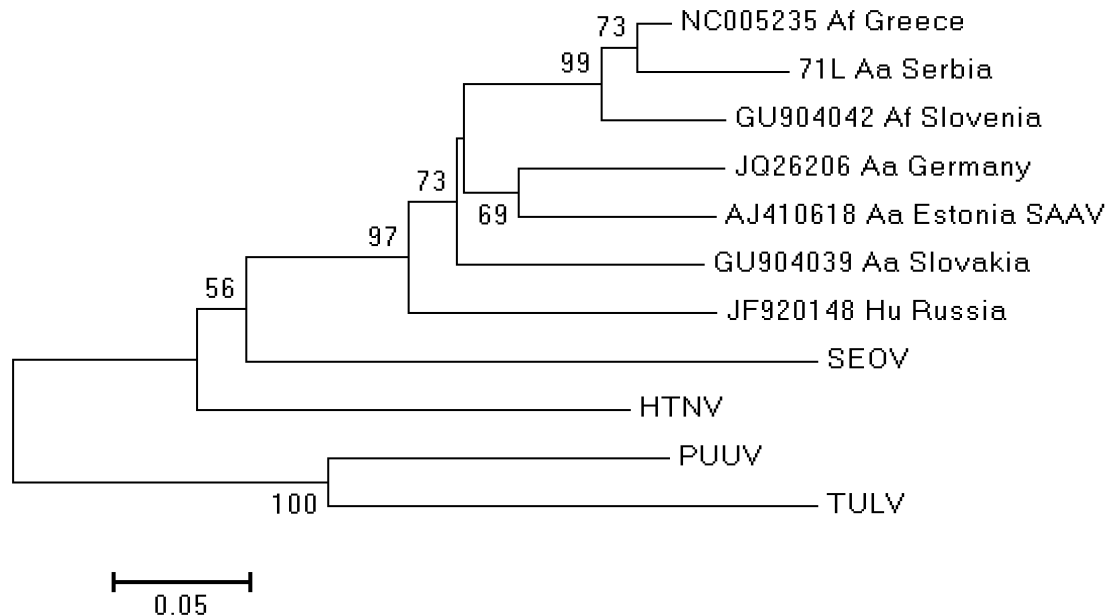
The obtained L segment sequence was first analyzed by BLAST at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) in order to determine similarity scores to specific hantaviruses. Both data sets for L and S segment of DOBV were aligned using CLUSTAL W implemented in the MEGA 5.1 software package (<http://www.megasoftware.net>). Further analysis involved jModeltest 0.1.1 software to define the best-fit nucleotide model of substitution, using all 88 proposed models (Posada, 2008). The best-fit models were found to be Transitional model 2 with a propor-

tion of invariant sites (TIM2 + I) and 3-parameter model 2 with gamma distributed rate heterogeneity and proportion of invariant sites (TPM2u + G + I), for the L and S segments of DOBV, respectively. To identify the obtained sequences and for further analysis of both data sets, phylogenetic trees were reconstructed in MEGA 5.1 software package. The topology of the reconstructed tree for the S segment data set was examined by a likelihood-mapping algorithm implemented in the TREE-PUZZLE V5.2 software package, without computing an overall tree (Schmidt et al., 2002). Namely, this algorithm examines the support of internal branches based on quartets of randomly selected sequences from a data set. Further phylogenetic analysis was done using PAUP version 4.0b1 (Swofford, 1998).

In order to examine possible recombination in the alignment of the S segment data set, three different software packages were employed, including Bootscan, as implemented in Simplot, Recombination Detection Program 3 (RDP3) and SplitsTree4 V 4.12.6 (Lole et al., 1999; Huson and Bryant, 2006; Martin et al., 2010). Bootscan analysis was applied according to the phylogenetic tree obtained for the S segment data set. Briefly, the sequences used in this analysis were grouped according to their clustering on the phylogenetic tree. Therefore, the S segment data set was divided into eight different groups, matching the corresponding clusters on the tree. Bootscan analysis was performed with a Kimura (2-parameter) distance model, a window size of 160 bp and step size of 20 bp, and the 70% bootstrap value was defined as the cutoff level. For further investigation of potential recombination events, the same data set was examined in RDP3 software using seven algorithms RDP, MaxChi, Chimaera, GENECONV, BootScan, SiScan and 3Seq with a default model as suggested in the manual. In addition, the analysis of potential recombination was conducted in SplitsTree4 V 4.12.6 using the closest substitution model to that described above.

## RESULTS

Based on the partial L segment sequence the ob-



**Fig. 1.** Neighbor-joining (NJ) phylogenetic tree generated by MEGA5 software based on 303 nt, L segment of 7 examined DOBV sequences. The tree was rooted with reference strains of PUUV, TULV, SEOV and HTNV.

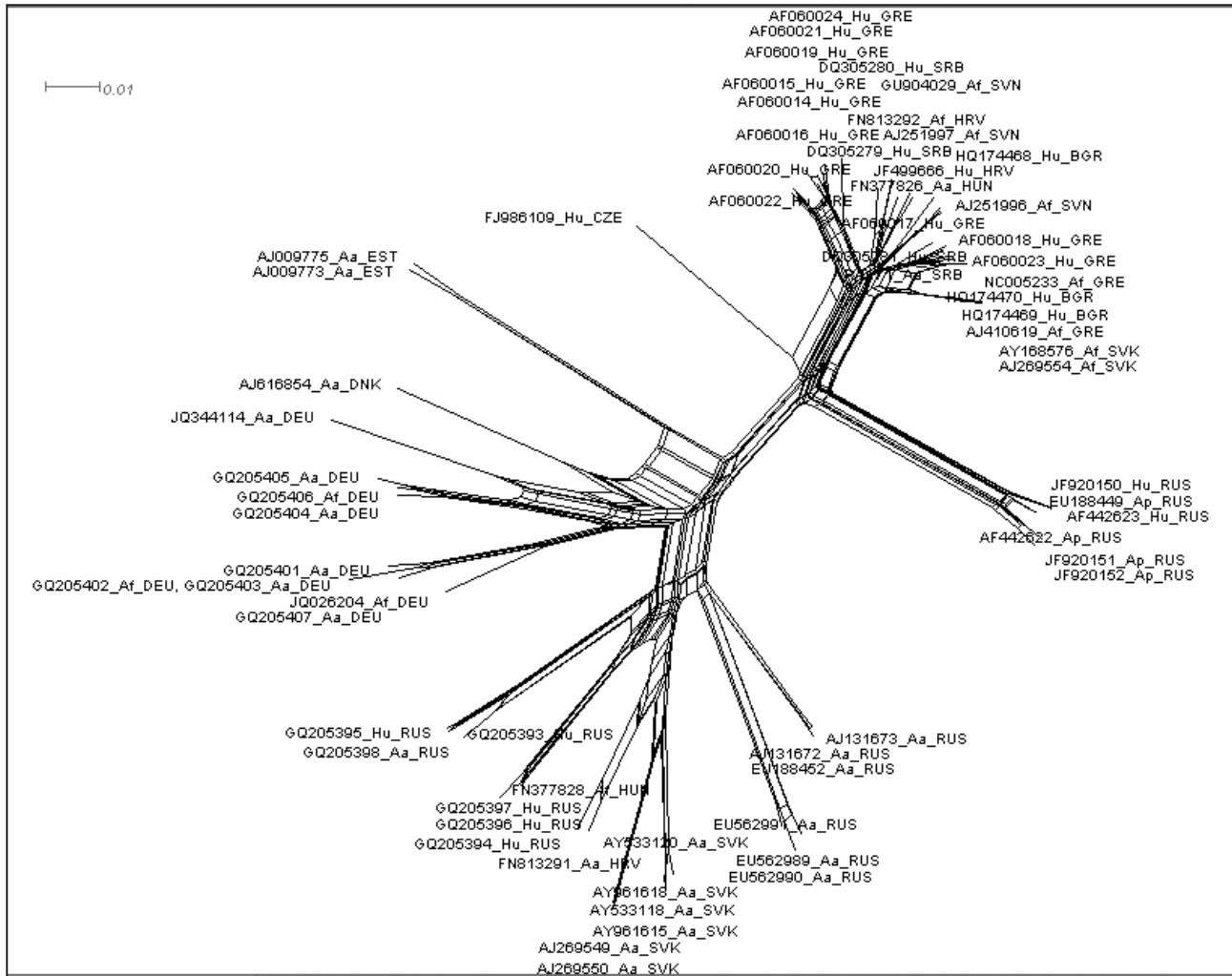
tained isolate was identified as DOBV, according to blast analysis. Detailed phylogenetic analysis of this sequence was further performed with the data set of six DOBV L segment sequences of a corresponding region, existing in the GenBank database, together with hantavirus reference strains. The length of the examined L segment sequence alignment was 303 nt, corresponding to position 2999-3301 nt of the DOBV L segment reference sequence (NC005235). This corresponds to 101 aa (988-1088aa) of the L protein. The mean nucleotide distance among all DOBV sequences included in the study was 15.6% (range 5.9-20.1%; SD 3.9). Molecular analysis of the L segment sequence revealed 18 different point mutations, but no evidence of changes in the aa chain when both sequences are compared with the corresponding nucleotide and amino acid reference strain (NC005235).

In the neighbor-joining (NJ) phylogenetic tree constructed by MEGA5 software package, the newly detected sequence was placed together with two sequences derived from *A. flavicollis* with 99% boot-

strap support (Fig. 1). A separate cluster contained other sequences isolated from *A. agrarius*.

The obtained alignment of the 528 nt S segment data set included one newly detected sequence from Serbia together with 66 DOBV sequences retrieved from GenBank. Prior to phylogenetic analysis, all sequences included in this alignment were screened for recombination. Bootscan analysis, implemented in Simplot, did not identify any positive recombination signal in the studied S segment region, similar to the other two recombination analysis softwares applied, RDP3 and SplitsTree4. The latter program utilizes sequence alignment to build recombination networks, illustrating the evolutionary relationships among examined taxa in the presence of recombination (Fig. 2). The results revealed no clear evidence of phylogenetic conflicts within the analyzed sequences.

Further phylogenetic analysis was performed on the 528 nt alignment of S segment sequences (387-914 nt according to S segment reference strain NC005233). Corresponding N protein sequences

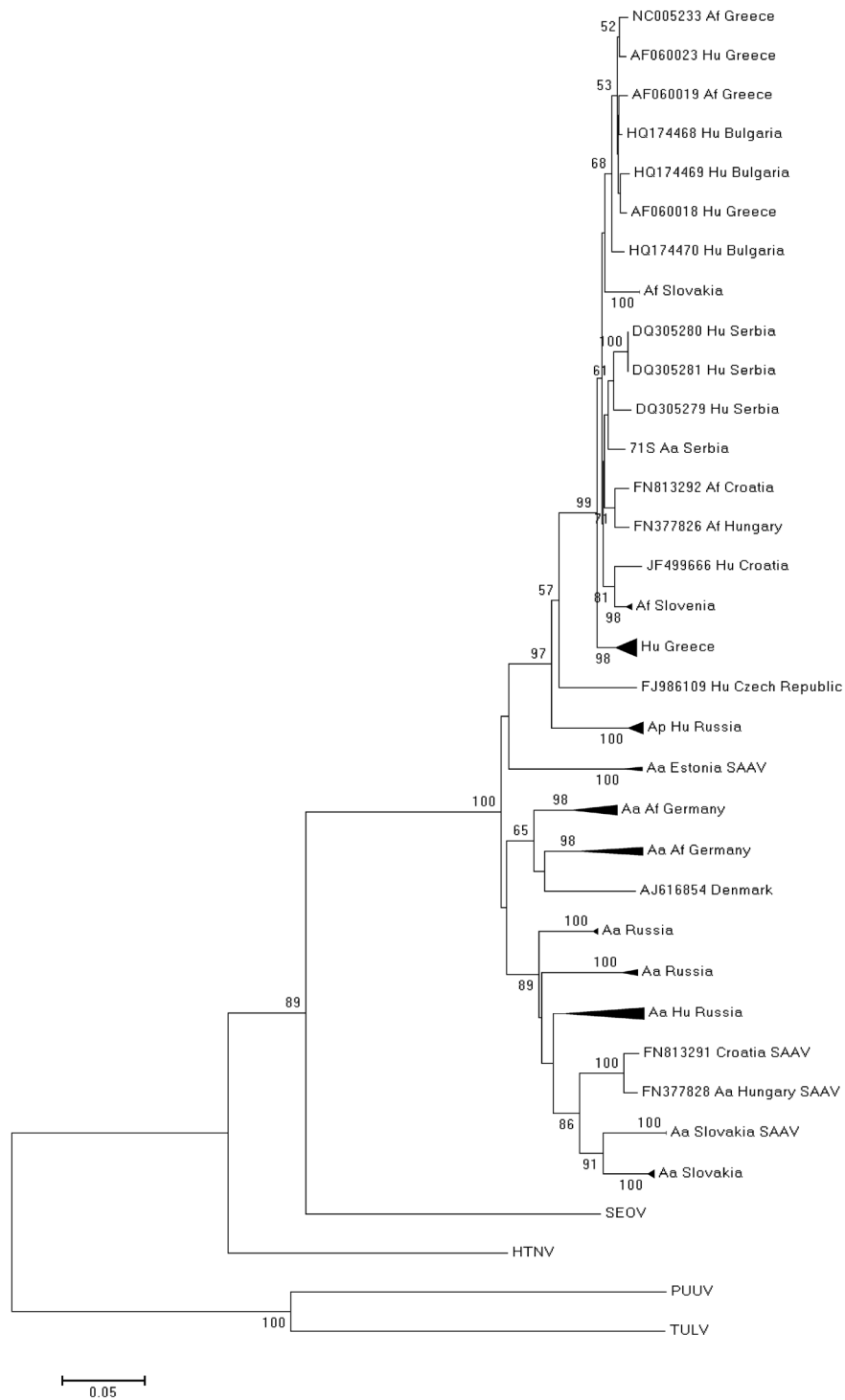


**Fig 2.** SplitsTree analysis of 528nt of 67 DOBV sequences. Networked relationships among the viral sequences do not significantly support the presence of potential recombination.

comprise 176 aa (positions 118-293). The mean nucleotide distance among all DOBV S segment sequences was 10.7% (range 0-16.5%; SD 4.2). Comparison of all Serbian sequences included in the study (three strains previously isolated from humans and one newly detected sequence isolated from *A. agrarius*) showed a nucleotide divergences of 1.7% (range 0-2.6%; SD 0.9). The studied S segment region of the newly detected sequence comprised 15 point mutations in comparison to the reference sequence (NC005233). All these nucleotide substitutions were synonymous, since no changes in the a strain were observed.

The neighbor-joining (NJ) phylogenetic tree included 67 DOBV S segment sequences divided into two clades, including DOBV-*Af* with DOBV-*Ap* and DOBV-*Aa* with 100% bootstrap support (Fig. 3). The tree was rooted using other hantaviral reference strains as outgroup (HTNV, SEOV, PUUV and TULV). The Serbian strain was placed in the DOBV-*Af* clade and it clustered together with three human isolates from Serbia.

The topology accuracy of the phylogenetic tree constructed for the S segment data set was inferred by using the TREE PUZZLE method. The results of



**Fig. 3.** Neighbor-joining (NJ) phylogenetic tree generated by MEGA5 software based on 528 nt S segment of 67 examined DOBV sequences. For better viewing, clusters of phylogenetically closely related sequences were compressed to triangles (size proportional to the number of sequences). The tree was rooted with reference strains of PUUV, TULV, SEOV and HTNV.

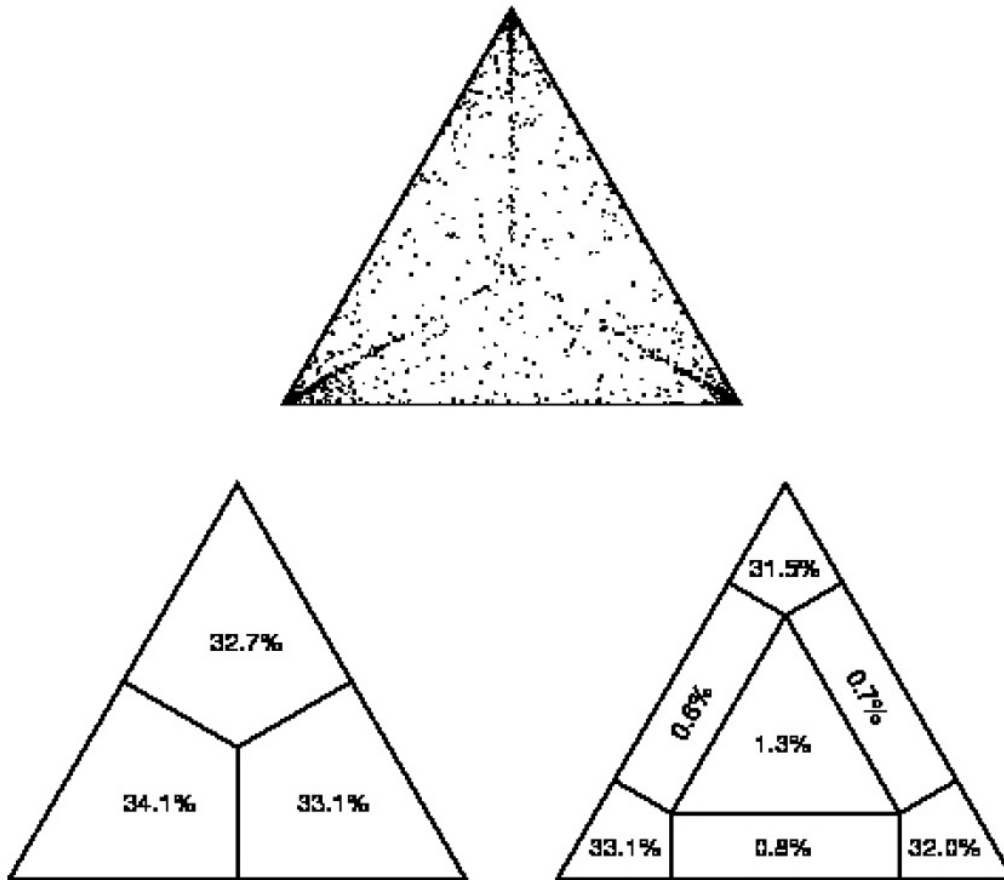


Fig. 4. Likelihood mapping analysis using a quartet grouping of 67 528 nt S segment sequences of DOBV. Number of quartets in the corners represents fully resolved phylogeny, whereas number of quartets in the center represents phylogenetic noise.

likelihood-mapping analysis support tree-like evolution. Most of the random quartets in the examined alignment were equally distributed in the three corners of the triangle (Fig. 4). Only 1.3% of the quartets were in the center of the triangle, representing unresolved quartets.

#### DISCUSSION

DOBV is known to be endemically present in Europe (Papa, 2012). Since its first detection in Serbia in 1992, the occurrence of this virus was demonstrated in almost all countries on the Balkan Peninsula (Gligic 1992; Papa, 2012). The presence of DOBV was reported in all republics of the former Yugoslavia, including Bosnia-Herzegovina, Slovenia, Croatia

and Montenegro (Hukic et al., 1996; Avsic-Zupanc et al., 2000; Gledovic et al., 2008; Nemeth et al., 2011; Papa and Christova, 2011). The occurrence of hantaviruses on the territory of Serbia has been shown in previous studies (Gligic et al., 1988; Gligic et al., 1989). Based on serological methods, hantaviruses were detected in *A. flavicollis*, *A. agrarius*, *Apodemus sylvaticus*, *Mus musculus*, *Clethrionomys glareolus* and *Microtus subterraneus*. Using molecular methods, the presence of DOBV was confirmed in human isolates from Serbia (Papa et al., 2006).

The studied region of the DOBV L segment is part of the conserved region described in all hantaviruses (Nemirov et al., 2003). This conserved region includes six different motifs (premotif A and motifs

**Table 1.** PCR primers used for amplification of DOBV S and L segments

Name	sequence (5'-3')	Nucleotide position	Genome segment
DOB-S-F1	GTAGTAGGCTCCCTAAAAAGC	3-23nt	S
DOB-S-R1	GGGATTACATAAAGCATGGGA	1343-1363	S (3-1363nt)
DOB-S-F2	CACTACACTAAAGATGGCAA	23-42	S
DOB-S-R2	GGATAATGCAACAAATACAATTA	1323-1345	S (23-1345nt)
HAN-L-F1	ATGTAYGTBAGTGCWGATGC	2936-2946	L
HAN-L-R1	AACCADTCWGTYCCRTCATC	3368-3387	L(2936-3387nt)
HAN-L-F2	TGCWGATGCHACIAARTGGTC	2947-2967	L
HAN-L-R2	GCRTCRTCWGARTGRTGDGCAA	3315-3336	L(2947-3336nt)

A, B, C, D, E), responsible for several activities. The sequence analyzed in our study is contained within motif B, which is responsible for the positioning of the template and primer relative to the active site.

The reconstructed neighbor-joining (NJ) phylogenetic tree, based on the 303 nt L segments of 7 examined DOBV sequences, compared sequences originating from different European countries, including Greece, Slovenia, Slovakia, Germany, Russia, Estonia and Serbia (Fig. 1). In the NJ tree, the newly detected sequence, isolated from *A. agrarius*, was placed together with sequences isolated from *A. flavicollis* with 99% bootstrap support. The other three sequences isolated from *A. agrarius* formed a distinct clade on the phylogenetic tree. The positioning of the Serbian sequence on the phylogenetic tree could possibly reflect local host switching of DOBV between *A. flavicollis* and *A. agrarius*. This possibility is supported by the fact that these two *Apodemus* species are known to share the same habitat. In addition, previous studies have already described spillover for DOBV and TULV (Schlegel et al., 2009; Schmidt-Chanasit et al., 2010; Schlegel et al., 2012). In this study, rodent identification was performed based on morphology. The two species in question (*A. flavicollis* and *A. agrarius*) are clearly morphologically distinct; however, genetic confirmation of species determination, based on the mitochondrial cytochrome b (cyt b) gene would be a useful complement.

Previous studies reported possible recombination events in DOBV and TULV (Sibold et al., 1999; Song et al., 2002; Klempa et al., 2003b). Screening for

recombination of the analyzed region in our study did not give positive results (Fig. 2).

As expected, on the phylogenetic tree constructed for the S segment data set, it is possible to distinguish two clusters (DOBV-*Af* vs. DOBV-*Ap* and DOBV-*Aa*) with 100% bootstrap support (Fig. 3). A similar topology of the phylogenetic tree was described in previous studies, with the exception for SAAV whose exact position depends on the length of studied S segment sequences (Klempa et al., 2005; Papa et al., 2006; Papa 2012; Schlegel et al., 2012). The first cluster contained sequences derived mostly from *A. flavicollis*, *A. ponticus* and humans. The topology of the illustrated tree indicates a possible monophyletic origin for DOBV-*Af* and DOBV-*Ap*. The only exception observed in this cluster was the newly detected sequence, isolated from *A. agrarius*. This sequence clustered with previously published human isolates from Serbia, indicating local geographical clustering. These strains were isolated from Serbian HFRS patients during a serious epidemic in Serbia and Montenegro that occurred in 2002 with 128 laboratory-confirmed cases (Papa et al., 2006). On the other hand, the DOBV-*Aa* cluster includes sequences isolated from *A. agrarius*, except three strains from Germany (GQ205402, GQ205406 and GQ20540). These strains may be considered a result of possible spillover (Schlegel et al., 2012).

In view of the fact that some clades on the phylogenetic tree based on DOBV S segment were supported by relatively low bootstrap values, likelihood-mapping analysis was performed to see whether the



illustrated tree was good enough to describe the evolution of the studied S segment region. The results obtained support the topology of the tree with a high percentage (98.7%) of fully and partially resolved quartets (Fig. 4).

In conclusion, this study provides insight into the phylogenetic relatedness of a newly detected sequence from Serbia and sequences retrieved from GenBank. The obtained results indicate local geographical clustering of DOBV sequences isolated from Serbia.

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