

# Molecular characterisation of rabies virus strains in the Republic of Macedonia

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**Abstract** Rabies, a worldwide zoonosis, remains a public-health concern despite oral wildlife vaccination in Europe. After a ten-year break, Macedonia reported eight rabies cases in 2011–2012. Two countries (Serbia and Bulgaria) bordering Macedonia are reporting cases in domestic and wild animals. This report describes the genetic characterisation of eight isolates from Macedonia compared with representative samples from neighbouring countries. All of the isolates tested belong to the Eastern European group, with a high degree of nucleotide sequence identity in the nucleoprotein gene. The close genetic relationship between isolates from the three bordering countries suggests that wildlife is responsible for rabies movements in the region.

**Keywords** Rabies virus · Republic of Macedonia · Balkans · Molecular epidemiology

## Abbreviations

N	Nucleoprotein
CE	Central Europe
EE	Eastern Europe
NEE	North-Eastern Europe
SF	Serbian fox
NJ	Neighbour-joining
WE	Western Europe
C	European part of Russia
D	Centre of the European part of Russia
RT-PCR	Reverse transcription polymerase chain reaction
FTA	Fast technology for analysis of nucleic acids
RV	Rabies Virus

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Rabies is caused by members of different virus species within the genus *Lyssavirus*, family *Rhabdoviridae*. Molecular studies have become a crucial tool for epidemiological investigations and for investigating the spread of the virus and species diversity. In Eastern Europe, at least four different variants have been described, belonging to the groups CE (Central Europe), EE (Eastern Europe), NEE (North-Eastern Europe) and SF (Serbian fox) [1, 5]. In July 2011, Macedonian veterinary authorities reported the first rabies case in the country [3]. A total of eight cases had been recorded by late February 2012. These new reported cases probably reflect a higher level of surveillance thanks to implementation of a rabies control program in the territory. Herein, we investigate the genetic characterisation of all eight isolates and several other isolates from surrounding countries to identify RV variants and phylogeographical relationships among the tested viruses found close to Macedonia (Serbia and Bulgaria). All three of these countries have ongoing wildlife vaccination campaigns against rabies using oral live attenuated vaccines (Bulgaria since 2009, Serbia since 2010 and Macedonia since 2011). In Macedonia, two oral vaccination campaigns were conducted throughout the territory in spring and autumn 2011 with SAD B19.

From January 2011 to the end of February 2012, 371 samples from different animal species (27 animals suspected of rabies and others collected for the national monitoring programme) were submitted to the rabies laboratory at the Faculty of Veterinary Medicine in Skopje for testing by the direct fluorescent antibody test [2], cell isolation test [10] and RT-PCR prior to genetic characterisation [6]. Of all the animals analysed, eight (four foxes and four wolves) were shown to be positive for rabies antigen, infectious virus and viral RNA. Viral RNA was extracted from a 10 % (v/w) brain homogenate of the six samples collected in 2011 and two samples isolated in 2012, stabilised on FTA cards [7]. A panel of samples stabilised on FTA cards was obtained from both Serbia (n = 6) and Bulgaria (n = 5) (Table 1).

The viral RNA was extracted from 200 µL of supernatant using an Iprep<sup>TM</sup> PureLink Virus Kit (Invitrogen, France) according to the manufacturer's instructions and subjected to partial nucleoprotein (N) gene amplification

(positions 55 to 660) by hnRT-PCR as described previously [6]. Following amplification, the PCR products were sequenced bi-directionally, using the same primers, by Beckman Coulter Genomics (Takeley, United Kingdom). A dataset of 97 sequences was constituted for the N gene analysis, including the eight sequences recently obtained from Macedonia, 10 sequences from Bulgaria, 24 from Serbia and 55 others referenced N gene sequences (Table 1).

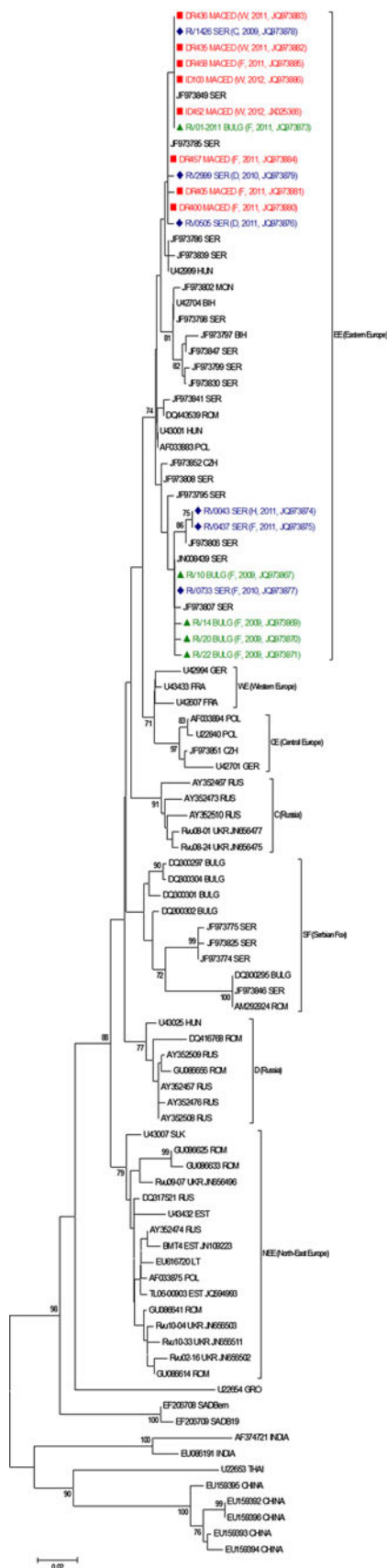
Phylogenetic analysis was performed using the neighbour-joining (NJ) method (Kimura 2-parameter) with MEGA software version 5 [9]. The bootstrap probabilities of each node were calculated using 1,000 replicates to assess the robustness of the NJ method.

The NJ tree (Figure 1) shows that all of the tested isolates from Serbia, Bulgaria and Macedonia during 2010-2012 correspond to field isolates (i.e., rabies virus), while the SAD sequences (EF206709 and EF206708) included in the study belong to SAD group (bootstrap support of 98). Ten percent divergence was found between SADB19 and the isolates of group EE. This demonstrated that no vaccine-induced rabies cases occurred during vaccination campaigns in Macedonia. Phylogenetic analysis of the N gene demonstrated that the eight field isolates from the Republic of Macedonia belong to the cosmopolitan lineage, more specifically, the EE group. All eight strains are closely related, with 99.6 % nucleotide sequence identity to each other. The NJ tree also confirms the circulation of different groups in Bulgaria: EE and SF groups, as previously shown by McElhinney et al. [5]. All six isolates from Serbia belong to the EE group. Figure 2 shows the geographical distribution of all the groups identified in this study.

Group EE consists of the eight Macedonian sequences and different sequences from Southeast Europe: Czech Republic, Serbia, Bulgaria, Hungary, Montenegro, Bosnia-Herzegovina, Romania and Poland. Referenced Eurasian sequences included in the phylogeny clustered with the CE, WE, NEE, C, D and SF groups. The CE sublineage encompasses sequences from Central Europe, group WE contains isolates from Western Europe, and group NEE contains sequences from North-Eastern Europe and Russia (Figure 1). Group C contains Russian and Ukrainian sequences, while group D encompasses sequences from

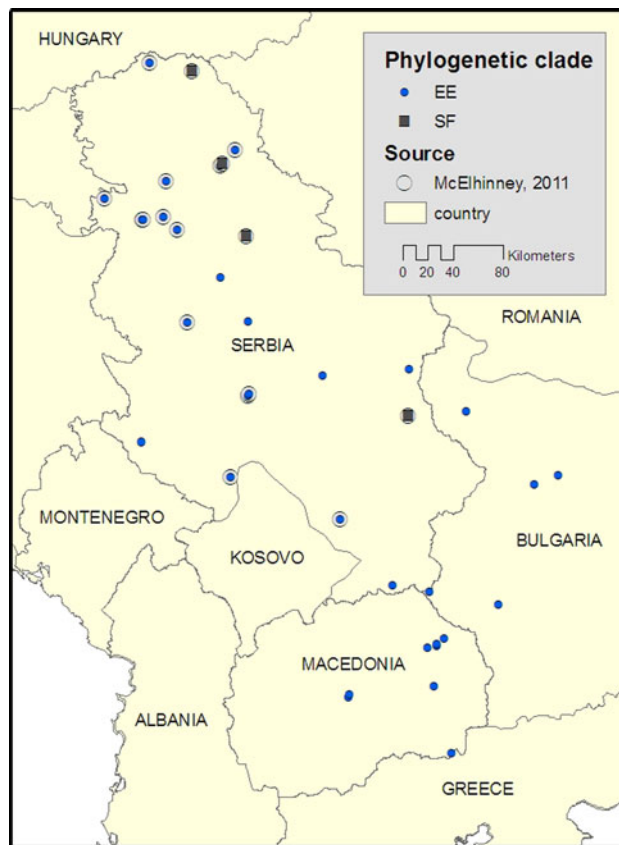
**Table 1** Description of isolates used in this study by country and by species

Number of tested isolates					
Country	<i>Vulpes vulpes</i>	<i>Canis lupus</i>	<i>Felis catus</i>	<i>Canis lupus familiaris</i>	<i>Equus ferus</i>
Republic of Macedonia	4	4			
Serbia	2		1	2	1
Bulgaria	5				
Total	12	4	1	2	1



**Fig. 1** Neighbour-joining phylogenetic tree comparing the eight isolates from the Republic of Macedonia with representative sequences from Southeast Europe, Central, North-Eastern and Western Europe and Russia. The NJ tree is based on an analysis of the first 358 nt of the N gene, using the NJ method (1,000 replicates). Bootstrap values over 70% are shown next to the branches. The city, species (F, red fox; W, wolf; C, cat; D, dog; H, horse; J, jackal), year of sample collection from Macedonia, Bulgaria and Serbia, and GenBank accession no. are included for each taxon. The previously assigned lineages (Eastern Europe [EE], North-Eastern Europe [NEE], Central Europe [CE], Western Europe [WE]) [1], groups C (European part of Russia) and D (centre of the European part of Russia) [4], and group SF (Serbian fox) [5] are indicated on the tree. Abbreviations: GER, Germany; AUT, Austria; BIH, Bosnia and Herzegovina; BUL, Bulgaria; CZH, Czech Republic; EST, Estonia; FRA, France; HUN, Hungary; LAT, Latvia; LT, Lithuania; MON, Montenegro; POL, Poland; ROM, Romania; UKR, Ukraine; RUS, Russia; SERB, Serbia; SLN, Slovenia; SLK, Slovak Republic; YOU, Bosnia and Herzegovina

Russia [4], Romania and Hungary. Two variants were circulating in Serbia within the EE and SF groups. Group SF, which comprised published sequences from Bulgaria,



**Fig. 2** Map of Southeast Europe showing the location of the eight RV samples from the Republic of Macedonia and from neighbouring countries according to the results of the phylogenetic analysis of the N gene. Samples are given symbols according to the defined lineage: (EE [Eastern Europe], D [centre of the European part of Russia] and SF [Serbian fox])

Serbia and Romania, was linked to the cluster of European viruses (EE, CE and WE) and groups C and D (limited bootstrap support of 25 %).

Perfect nucleotide sequence identity (100 %) was found between four samples from Macedonia (fox DR458 and three wolves, DR435, DR436 and ID103, all located between 14 and 25 kilometres from the Macedonian-Bulgarian border), one sample isolated in 2011 in Serbia (cat RV2011-1426\_SER, found 7 kilometres from the Macedonian border), and a published Serbian isolate (JF973849). Within group EE, there was less than 1.5 % divergence between all isolates.

Foxes are found in almost all habitats, and there are no natural barriers preventing rabies spreading in bordering areas of Serbia, Bulgaria and Macedonia [8]. The Macedonian-Serbian and Macedonian-Bulgarian borders are in mountainous areas (maximum altitude around 1700 m and 1900 m, respectively). This study is the first report on RV circulation coupled to genetic analysis of RV strains in the Republic of Macedonia. The landscape, which is globally less than 1000 m above sea level and favorable for fox habitats, can explain why all of the isolates tested from Macedonia were resolved in the EE group, comprising isolates from Southeast Europe, Bulgaria and Serbia. The close genetic relationship between isolates from Macedonia and viruses isolated recently from the neighbouring country of Serbia, near the border, suggests a cross-border rabies movement. The transmission of rabies virus by human-mediated animal movements, the translocation of infected animals, and the illegal movement of rabid domestic carnivores is well described and could also explain the movement of infected animals. A previous study suggested a southward movement of rabies in wildlife from Hungary, Serbia and Romania into Bulgaria [5]. Few studies have been undertaken on the molecular epidemiology of rabies viruses in the Balkans, and further investigations are needed to better understand the phylogeny and evolution of rabies virus isolates in this region.

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