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Molecular diversity and evolutionary history of rabies virus strains circulating in the Balkans

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Molecular studies of European classical rabies viruses (RABV) have revealed a number of geographically clustered lineages. To study the diversity of Balkan RABV, partial nucleoprotein (N) gene sequences were analysed from a unique panel of isolates (n=210), collected from various hosts between 1972 and 2006. All of the Balkan isolates grouped within the European/Middle East Lineage, with the majority most closely related to East European strains. A number of RABV from Bosnia & Herzegovina and Montenegro, collected between 1986 and 2006, grouped with the West European strains, believed to be responsible for the rabies epizootic that spread throughout Europe in the latter half of the 20th Century. In contrast, no Serbian RABV belonged to this sublineage. However, a distinct group of Serbian fox RABV provided further evidence for the southwards wildlife-mediated movement of rabies from Hungary, Romania and Serbia into Bulgaria. To determine the optimal region for evolutionary analysis, partial, full and concatenated N-gene and glycoprotein (G) gene sequences were compared. Whilst both the divergence times and evolutionary rates were similar irrespective of genomic region, the 95 % highest probability density (HPD) limits were significantly reduced for full N-gene and concatenated NG-gene sequences compared with partial gene sequences. Bayesian coalescent analysis estimated the date of the most common recent ancestor of the Balkan RABV to be 1885 (95 % HPD, 1852–1913), and skyline plots suggested an expansion of the local viral population in 1980–1990, which coincides with the observed emergence of fox rabies in the region.

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INTRODUCTION

Molecular studies of classical rabies virus (RABV) both at the national and global levels have broadened the understanding of the diversity of this virus species, which is responsible for an estimated 55 000 human deaths per year (WHO, 2005). In Europe, RABVs cluster within a 'Cosmopolitan lineage' having ancestral roots in Europe in the 18th century before its assumed widespread dispersal to Asia, Africa and the Americas as a result of European exploration and colonization (Smith *et al.*, 1992; Badrane & Tordo, 2001; Nadin-Davis & Bingham, 2004; Bourhy *et al.*, 1999; McElhinney *et al.*, 2006). More comprehensive phylogenetic analysis of European RABV strains revealed a number of distinct groups, each associated with a particular geographical area, suggesting that RABV has spread westwards and southwards across Europe during the last century (Bourhy *et al.*, 1999). Rabies is known to have occurred in South Eastern Europe in both domestic animals and wildlife since the Middle Ages (reviewed by Mutinelli *et al.*, 2004). Whereas canine rabies was predominant at the beginning of the 20th century and eventually controlled in the 1960s, fox-mediated (sylvatic) rabies steadily increased after World War II, becoming a major public health problem in recent decades (Mutinelli *et al.*, 2004; Petrovic 1987; Wandeler, 2004).

In 1977, fox rabies entered northern Serbia from Hungary and Romania (Lontai, 2004), spreading slowly towards the south. Physical barriers such as the Sava and Danube rivers had contained the fox rabies epidemic for some

Supplementary material is available with the online version of this paper.

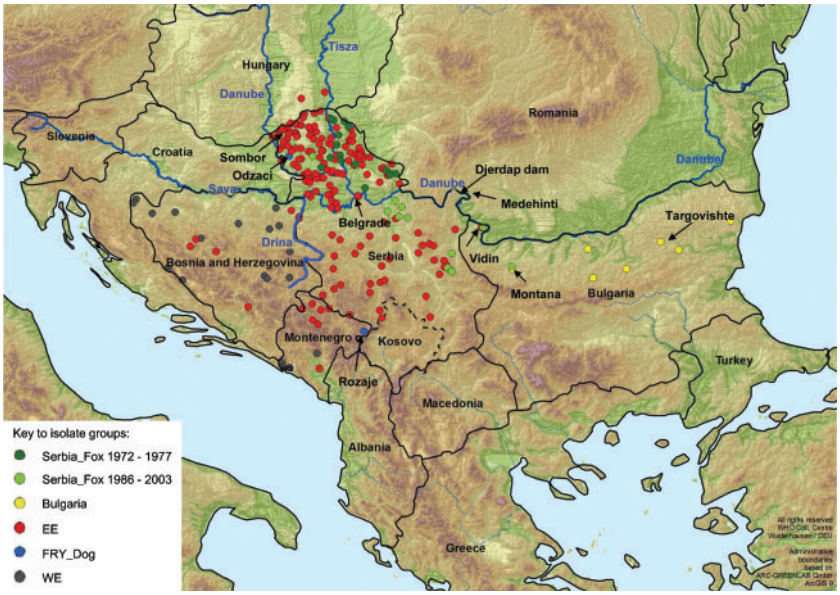

Fig. 1. Map of Serbia and neighbouring countries, including approximate locations and distribution of the RABV variants determined by phylogenetic analysis of the N gene (N400 bp). The inclusion of the Serbian 'FRY dog' variant RV1146 was based on partial G-gene data.

time (Fig. 1), but were eventually crossed, resulting in the first noted cases of fox rabies in Central Serbia in 1986 within a region previously free of canine rabies. By 1998, the epizootic had reached Kosovo. Although limited field trials on oral rabies vaccination (ORV) of foxes were initiated in Serbia in 1999 (Mutinelli *et al.*, 2004), the number of reported rabies cases in Serbia increased (data extracted from Rabies Bulletin Europe, Table 1). Croatia and Bosnia & Herzegovina (BiH) recorded the incursion of the fox rabies epizootic from their northern borders in 1977 and 1982, respectively (Velic & Sandrac, 2007). The conflict in the Balkans region, between 1991 and 1995, resulted in a failure to implement adequate rabies control programmes, which is reflected in the steady rise in rabies cases from 1992 (Table 1).

Very few comprehensive studies have been published on the molecular epidemiology of RABV in West Balkan countries (McElhinney *et al.*, 2006; Johnson *et al.*, 2008). A small-scale study was previously undertaken on 32 RABV from the Former Republic of Yugoslavia (FRY) by both genetic and mAb typing (Stankov, 2001), indicating the existence of at least two independent cycles of fox-associated RABV strains. The EU is currently co-financing ORV programmes with an overall aim to eliminate terrestrial rabies from the Balkans (Freuling *et al.*, 2008). As such a more detailed epidemiological picture of the circulating RABV variants is essential to inform vaccination strategies.

Therefore, the objective of this study was to gain more insights into the phylogeny and evolution of rabies virus isolates from the West Balkans. Here, we present a study undertaken on a significantly larger panel of isolates (n=210) originating from BiH, Montenegro and Serbia from a range of hosts between 1972 and 2006. Furthermore, nucleotide sequences from other RABV isolates were combined with the panel, enabling us to determine the extent of virus divergence and geographical distribution during an important period in the history of the region.

Estimating when viral lineages emerged or diverged relies on an accurate estimation of the rate of nucleotide substitution and subsequent application of a molecular clock. Bayesian techniques using the Markov Chain Monte Carlo (MCMC) methods have been successfully applied to RABV to estimate the evolutionary rate and divergence times from dated sequences (Hughes *et al.*, 2005; Talbi *et al.*, 2009, 2010; Ming *et al.*, 2010). We have applied a relaxed molecular clock to a variety of N- and G-gene datasets to obtain estimates of the time to the most recent common ancestor (TMRCA), rate of evolution and population dynamics for Balkan RABV.

Table 1. Reported rabies cases in Serbia and Montenegro 1993–2005 (Source Rabies Bulletin Europe)

Year/no.	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
Wildlife	53	25	100	81	67	72	84	112	185	182	184	163	264
Domestic	29	21	18	29	31	31	29	40	60	36	48	39	107
Total	82	46	118	110	98	103	113	152	245	218	232	202	371

RESULTS AND DISCUSSION

All Balkan RABV sequences were resolved within the Cosmopolitan lineage (comprising Eurasia, Middle East, Africa 1, Africa 4 and vaccine strains), more specifically within the European/Middle East lineage (Fig. 2). No Asian, Arctic, Arctic-like or bat variants were identified in our study panel.

The West European (WE) sublineage represented on the phylogenetic tree (Fig. 2) encompasses previously published viral sequences from France, Austria, Belgium, Germany, Slovenia, BiH and Montenegro, demonstrating the widespread distribution of this variant in Europe during the fox epizootic in the latter half of the 20th Century (Bourhy *et al.*, 1999). Our data confirm the presence of the WE variant in the West Balkans. The majority of RABV isolates from BiH and four isolates from Montenegro (RV1241, RV1260, RV1261 and RV1282) joined the WE cluster (represented by the RV1189 group [VI] in Fig. 2). The Bosnian isolates in the WE sublineage are similar to the previously published 1986 BiH fox isolate (86111YOU, GenBank accession no. U42706), despite being collected over two decades (1986–2006) (Kissi *et al.*, 1995). The four isolates from Montenegro in group VI are identical to the two isolates from BiH (400 nt, N-gene), despite their origins in the far north of Bosnia.

A single published sequence from a 1972 fox isolate from FRY (86106YOU, GenBank accession no. U22839) was identified within the WE group (Bourhy *et al.*, 1999). However, upon further enquiries with the Pasteur Institutes at Novi Sad and Paris, it was discovered that the 86106YOU isolate was originally sample 2924/72 (designated RV1147 at AHVLA), which in our study was typed as a Serbian fox (SF) variant. We therefore excluded 86106YOU from our study, believing it to be erroneous. Hence, no WE variants were identified within Serbia, despite the large numbers of Serbian RABV isolates included in this study.

The majority of the Balkan RABV isolates in our study were resolved in the East European (EE) sublineage, comprising isolates from Hungary, BiH, Serbia, Montenegro, Bulgaria, Poland and the Czech Republic, collected from various species between 1977 and 2006. Within EE, the viruses clustered mainly geographically, but occasionally a small group of chronologically related viruses were apparent. The largest numbers of identical sequences were obtained for groups III, IX and X. Group III, represented by RV1157, comprised 20 isolates collected between 1977 and 2000 in northern Serbia, particularly in Sombor. Thirteen of the group III isolates were collected during the 1977 fox rabies epizootic that had moved into northern Serbia from Hungary and Romania. It is clear from this group that some viral strains can persist for a considerable length of time as group III strains were collected over four decades. Group IX isolates (n=22), represented by RV1198, were collected in northern Serbia between 1997 and 2006. The group X isolates (n=18), represented by RV1208, were collected from northern Serbia in 1997, 1999 and 2000. Although the largest number of this group were collected from cats (n=10), the inclusion of six fox isolates would suggest that the feline rabies cases were fox-mediated spill-over events. The EE viruses which were previously shown to be antigenically divergent by mAb typing (mAb groups I and II) and spatially separated (Stankov, 2001) were further resolved on the N400 phylogenetic tree (Fig. 2).

A single Austrian isolate (RV1339) fell central to a large cluster of Serbian isolates within EE. This isolate was collected in 2001 from a rabid dog imported from Belgrade into Austria. A fox isolate (RV2157) collected from Belgrade in 2002 was 100 % identical to the Austrian imported case in the N400 nucleotide region. Interestingly, an earlier case imported into Austria in 1999 involved a rabid horse from Serbia. This isolate (RV1538) also clustered in the EE sublineage but the closest sequence (99 % identity) belonged to a dog isolate from Bosnia (RV1188), which was identical to a Bosnian Wolf strain (8653YOU, GenBank accession no. U42704) both isolated in 1986. Such results retrospectively emphasize the importance of genetic characterization as a tool for tracing imported rabies cases.

No isolates within our Balkan panel joined the North-east European (NEE) sublineage, suggesting that this virus variant may not have established in Serbia, BiH and Montenegro. A recent phylogenetic study of Romanian RABV (Turcitu *et al.*, 2010) reported the presence of a number of viruses widely dispersed through Romania, which aligned closely with the NEE sublineage. The occurrence of the NEE variant so far south may have resulted from a Westward incursion from Ukraine or Moldova. However, a NEE isolate (94250SLK, GenBank accession no. U43007) had previously been identified in Slovakia (Bourhy *et al.*, 1999) and therefore it is plausible that the variant moved into Romania from the North. The NEE variant has been particularly associated with raccoon dogs (*Nyctereutes procyonoides*) in North-west Russia and North-east Europe (Kuzmin *et al.*, 2004). The distribution of the non-indigenous raccoon dog is expanding to the West and South East of Europe and may hinder rabies vaccination control programmes targeted towards foxes (Singer *et al.*, 2009; Ćirović, 2006). Raccoon dogs were first recorded in Serbia in 1978 (Ćirović & Milenković, 1999) and have been associated with a single case of rabies in 2002 (Rabies Bulletin Europe); however, the virus isolate was not available for study. Insufficient data are available for Hungary and Ukraine to make any firm conclusions about the direction in which the NEE variant dispersed.

A group of 'SF' RABV isolates and a previously published fox isolate (86107YOU, GenBank accession no. U42703) collected between 1972 and 1977 were resolved on the tree with significant bootstrap support (100 %) and represented the clearest chronological and geographical segregation (Fig. 2). The majority of the 'SF' RABV were collected in northern and central Serbia. However, the early SF isolates (1972–1977) were clearly limited in their distribution to northern Serbia (represented by dark green circles in

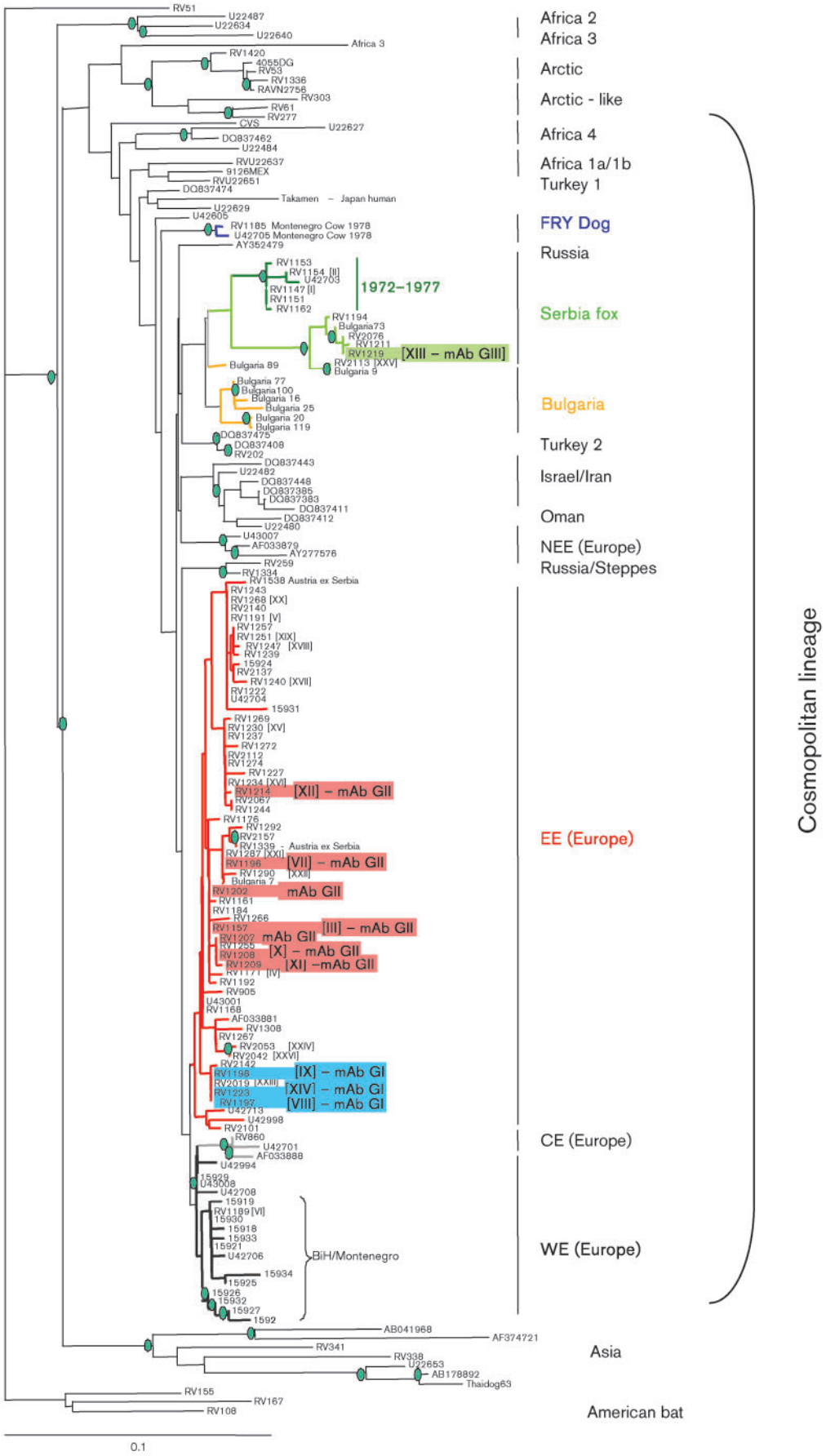
RV51
U22487
U22634
U22640
Africa 3
RV1420
4055DG
RV53
RV1336
RAVN2756
RV303
RV61
RV277
CVS
U22627
DQ837462
U22484
RVU22637
9126MEX
RVU22651
DQ837474
Takamen – Japan human
U22629
U42605
RV1185 Montenegro Cow 1978
U42705 Montenegro Cow 1978
AY352479
RV1153
RV1154 [II]
U42703
RV1147 [I]
RV1151
RV1162
1972–1977
RV1194
Bulgaria73
RV2076
RV1211
RV1219
[XIII – mAb GIII]
RV2113 [XXV]
Bulgaria 9
Bulgaria 89
Bulgaria 77
Bulgaria100
Bulgaria 16
Bulgaria 25
Bulgaria 20
Bulgaria 119
DQ837475
DQ837408
RV202
DQ837443
U22482
DQ837448
DQ837385
DQ837383
DQ837411
DQ837412
U22480
U43007
AF033879
AY277576
RV259
RV1334
RV1538 Austria ex Serbia
RV1243
RV1268 [XX]
RV2140
RV1191 [V]
RV1257
RV1251 [XIX]
RV1247 [XVIII]
RV1239
15924
RV2137
RV1240 [XVII]
RV1222
U42704
15931
RV1269
RV1230 [XV]
RV1237
RV1272
RV2112
RV1274
RV1227
RV1234 [XVI]
RV1214
[XII] – mAb GII
RV2067
RV1244
RV1176
RV1292
RV2157
RV1339 – Austria ex Serbia
RV1287 [XXI]
RV1196
[VII] – mAb GII
RV1290 [XXII]
Bulgaria 7
RV1202
mAb GII
RV1161
RV1184
RV1266
RV1157
[III] – mAb GII
RV1202 mAb GII
RV1265
[X] – mAb GII
RV1208
RV1209
[XII] –mAb GII
RV1171 [IV]
RV1192
RV905
U43001
RV1168
AF033881
RV1308
RV1267
RV2053 [XXIV]
RV2042 [XXVI]
RV2142
RV1198
[IX] – mAb GI
RV2019 [XXIII]
RV1223
[XIV] – mAb GI
RV1197
[VIII] – mAb GI
U42713
U42998
RV2101
RV860
U42701
AF033888
U42994
15929
U43008
U42708
15919
RV1189 [VI]
15930
15918
15933
15921
U42706
15934
15925
15926
15932
15927
1592
BiH/Montenegro
AB041968
AF374721
RV341
RV338
U22653
AB178892
Thaidog63
RV155
RV167
RV108
0.1
Africa 2
Africa 3
Arctic
Arctic - like
Africa 4
Africa 1a/1b
Turkey 1
FRY Dog
Russia
Serbia fox
Bulgaria
Turkey 2
Israel/Iran
Oman
NEE (Europe)
Russia/Steppes
EE (Europe)
CE (Europe)
WE (Europe)
Asia
American bat
Cosmopolitan lineage

Fig. 2. Phylogenetic tree comparing 155 Serbian, BiH and Montenegrin RABV with representative global GenBank sequences using a 400 base sequence region of the N gene. Significant nodes, i.e. those with bootstrap values greater than 70 % of 1000 data replicates are indicated on the main branches as a green circle. mAb typing data (antigenic group I, blue; antigenic group II, red; antigenic group III, green) are indicated where available (Stankov, 2001).

Fig. 1). The natural barriers of the rivers Danube and Sava (Fig. 1), combined with relatively low fox densities in the South, are believed to have played an important role in restricting rabies distribution to the northern regions of Serbia, when the rabies epizootic in the late 1970s spread to Serbia from Hungary and Romania (Mutinelli *et al.*, 2004). Fox rabies was not recorded in Central Serbia until 1986 and the restricted geographical distribution may be reflected in the limited genetic diversity of isolates in this 'SF' group. This earlier 'SF' group was closely related to a distinct cluster of isolates (nine foxes, one cat and a wolf) collected between 1986 and 2003 from throughout Serbia and from Bulgaria. The clustering of north SF isolates collected in 1986 with isolates collected in central and east Serbia (in 2002) possibly reflects local evolution prior to the southwards spread of the epizootic. Isolated foci of infections were recorded in the late 1980s near to the joint borders of Serbia, Romania and Bulgaria. The surrounding territories in Serbia were rabies free and so the incursions were believed to have arisen due to rabid foxes crossing from Romania into Serbia via the Djerdap dam on the Danube river near Kladovo.

The 'SF' isolates above were more closely related to viruses from Bulgaria and the Middle East than European isolates, with the closest isolate (Bulgaria 89, GenBank accession no. DQ300302) collected in 2002 from a fox in the Eastern Province of Targovishte, Bulgaria. Two additional Bulgarian isolates Bulgaria 9 (wolf, Vidin, 2003, GenBank accession no. DQ300295) and Bulgaria 73 (fox, 2001, Montana GenBank accession no. DQ300300), grouped closely with the SF isolates (RV1194, RV1211, RV2076, group XIII and group XXV). These isolates originated from the Vidin and Montana regions of Western Bulgaria, which share a border with Serbia. In addition, a truncated analysis of available Romanian RABV sequences (Turcitu *et al.*, 2010) confirmed the presence of the 'SF' variant in a sheep collected in 2005 in Mehedinti, South West Romania (RO47, GenBank accession no. GU086629). This provides further evidence for the southwards wildlife-mediated movement of rabies from Hungary, Romania and Serbia into Bulgaria (Johnson *et al.*, 2007). The 'SF' virus isolates represented by RV1219 (group XIII, Fig. 2) had previously been reported as mAb group III variants (Stankov, 2001).

Two closely related viruses isolated from cattle in Rozaje, Montenegro in 1978 (RV1185 and 8658YOU), which remain distinct in Fig. 2, were previously shown to be more closely related to the dog and jackal variants from Georgia, North-east Turkey and the Middle East than the other variants identified in the Balkan region (EE, WE and Serbia fox) and proposed as a spill-over event with a virus from a lineage which was established in the earlier part of the 20th century, before the epizootic in red foxes (Bourhy *et al.*, 1999). An additional viral isolate (RV1146) collected from a dog in 1971 from Odzaci in northern Serbia has been determined to be identical to RV1185 in a partial G-gene region (data not shown) further supporting the theory that the cattle isolates in Montenegro resulted from spill-over events involving a possibly extinct ancestral dog variant (FRY dog).

The maximum clade credibility (MCC) tree for 67 Cosmopolitan RABV (N1350) obtained using BEAST (ESS >160) had a similar topology (Fig. 3) to that observed for the partial N400 phylogenetic tree (Fig. 2), but with improved support at the nodes (posterior probability values >80 % indicated for the key nodes). The estimated dates of the most recent common ancestor (MRCA) are illustrated for the key sublineages (when supported) with their 95 % highest probability density (HPD) dates (Fig. 3). The TMRCA for the Cosmopolitan panel (n=67), which includes European, Middle East, Africa 1 and Africa 4 RABV is estimated to be 161 years (95 % HPD, 111–213 years), which equates to the year 1844 (95 % HPD, 1792–1894). The TMRCA for the Balkan RABV including EE, WE, SF and FRY dog variants is estimated to be 120 years (95 % HPD, 91–157 years), which equates to the year 1885 (95 % HPD, 1848–1914). The mean rate of nucleotide substitution for the N-gene of the Cosmopolitan RABV (n=67) in this study, estimated using a Bayesian MCMC approach, was 3.889×10^{-4} substitutions per site per year (95 % HPD, $2.884–4.961 \times 10^{-4}$ substitutions per site per year). This is comparable to that observed for RABV in previous studies (Badrane & Tordo, 2001; Davis *et al.*, 2007; Talbi *et al.*, 2010; Ming *et al.*, 2010) despite differences in methodological approaches, confirming the strength of the available data (Drummond *et al.*, 2003).

The TMRCA and evolutionary rates (mean nucleotide substitution rates per site per year) of five sequence datasets for 37 Balkan RABV representing N (N400 and N1353), G (G600 and G1402) and concatenated (NG2755) sequences are provided in Table 3. Whilst both the TMRCA and evolutionary rates are similar irrespective of genomic region, the 95 % HPD limits are significantly reduced for complete N-gene and concatenated NG-gene sequences compared with partial N-gene and G-gene sequences (Table 3). The estimate of the date of the MRCA obtained for the Balkan RABV in the concatenated NG2755 MCC tree (Supplementary Fig. S1, available in JGV Online) is identical to that obtained for the expanded N1350 dataset (Fig. 3) albeit with slightly better confidence intervals (1885, 95 % HPD 1852–1913). Combining N- and G-gene data has previously been reported as optimally reflecting phylogenetic relationships of RABV (Bourhy

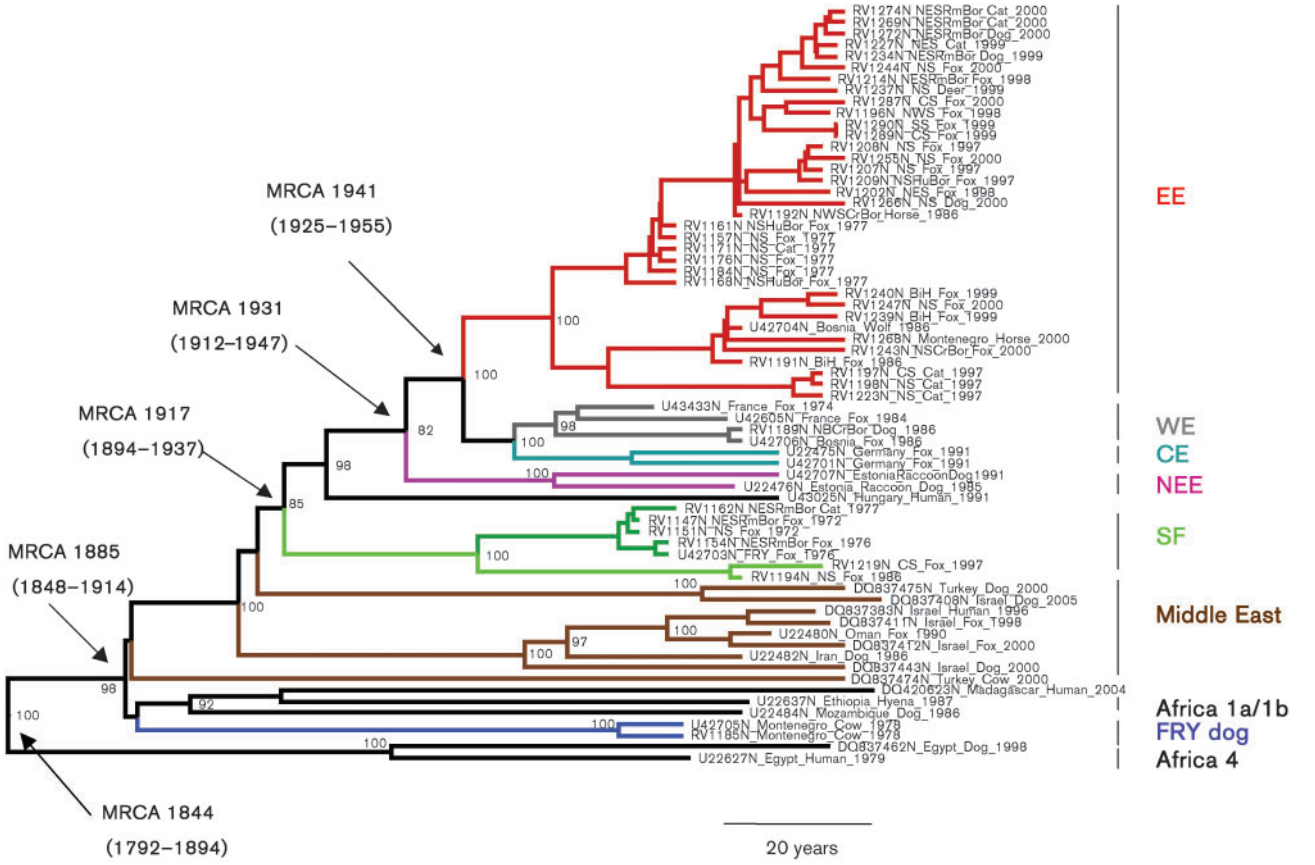

Fig. 3. MCC tree of 67 sequences of the Cosmopolitan lineage, derived from the N gene of RABV (1350 nt). The dates of the MRCA with 95 % HPD values are provided where supported at major tree nodes. Tip times reflect the year of sampling. Sublineages (FRY dog, WE, CE, EE, NEE, SF, Middle East, Africa 1 and 4) are indicated. Posterior probability values >80 % are included for the key nodes.

et al., 1999). The origins of the FRY dog ancestral variant estimated in this study are earlier than the previously reported early 20th century (Bourhy *et al.*, 1999), possibly due to the inclusion of a larger number of older sequences. The absence of the Middle East RABV in the NG2755 dataset may account for the slightly earlier estimate of the divergence time for the SF variant (1908, 95 % HPD 1884–1928, Supplementary Fig. S1) compared with the expanded N1353 analysis (1917, 95 % HPD 1894–1937, Fig. 3). The TMRCA obtained for the NEE variant in Fig. 3 (1931, 95 % HPD 1904–1942) and the known introduction of the non-indigenous raccoon dog into Eastern Europe between 1927 and 1957, may further strengthen the perceived importance of this species in the maintenance of this variant (Bourhy *et al.*, 1999). The ancestral origins of the WE, Central European (CE) and EE, estimated for both N-gene (Fig. 3) and concatenated NG-gene (Supplementary Fig. S1) sequences supports the belief of a dog to fox host switch in the early decades of the 20th Century.

Irrespective of the gene analysed (N or G), Bayesian skyline plots were suggestive of stable population sizes of Balkan RABV between 1930 and 1970. However, from 1970 a gradual decline in the effective population preceded a rapid expansion starting at ~1990 (Fig. 4a) for N-gene sequences (N1353) and ~1980 (Fig. 4b) for G-gene sequences (G1402). The depression observed may not be significant given the size of the confidence limits. The delay between the expansion observed for the two genes is represented by a biphasic increase for the concatenated Balkan sequences (NG2755, Fig. 4c). A truncated analysis of the Balkan EE sequences alone reflects a similar population profile (data not shown). The SF variant population appears stable over time but ESS values did not reach 100 due to the small numbers involved. The decline in the Balkan RABV population in the 1970s may have been due to the topographical restrictions (Danube and Sava rivers) and low fox densities. The emergence of infected foxes in Central Serbia in the mid 1980s and the subsequent widespread transmission throughout Serbia are reflected in the rapid expansion of the Balkan RABV population (Fig. 4a–c) and the increased number of reported cases in the region, which continued to rise until 2004 (Table 1). Whilst an absence of data during a particular period may

Table 2. Geographical and chronological distribution of isolates available in this study from FRY

Country	No. isolates	1970–1979	1980–1989	1990–1999	2000–2006
Bosnia & Herzegovina	21	0	4	3	14
Montenegro	13	1	0	2	10
Serbia	175	36	5	45*	89*
Kosovo	1	0	0	0	1
Total	210	37	9	50	114

*Includes imported case into Austria.

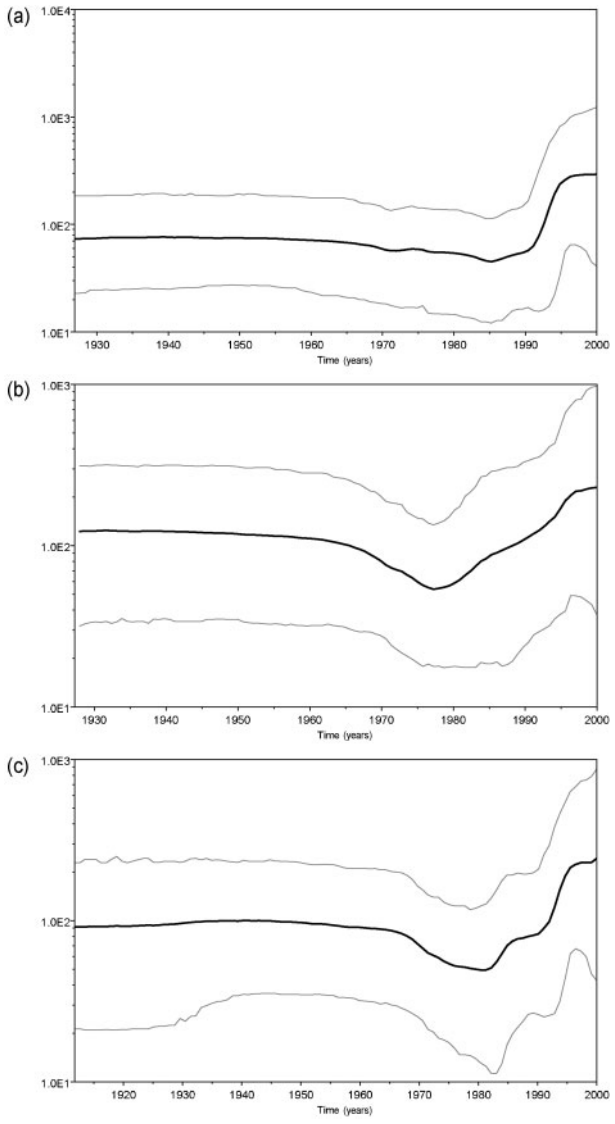

Fig. 4. Bayesian Skyline plots of population history for FRY RABV sequences (n=37) for (a) the N-gene sequences (1353 nt), (b) G-gene sequences (1402 nt) and (c) concatenated NG-gene sequences (2755 nt). The solid black line represents the mean values and the grey lines show the limits of the 95 % HPD. Time is shown in years across the bottom of the plot and the effective population size is shown on the left-hand scale.

result in a failure to observe the true dynamics at that time, clustering of samples in different time periods (e.g. greater numbers sampled in the 1970s and 2000s than during the 1980s and 1990s) should not introduce bias or artefacts (Rambaut *et al.*, 2008). Surveillance in Serbia, BiH and Montenegro during this period is believed to have been constant and effective, despite periods of unrest, thereby supporting an actual expansion of the RABV population.

Conclusions

This study represents the first comprehensive molecular epidemiological review of RABV in the West Balkans region. All of the RABV isolates investigated joined the Cosmopolitan lineage. The majority of the Serbian isolates were characterized as either belonging to the EE sublineage or the 'SF' group. However, a small number of divergent, possibly ancestral isolates were also resolved. Why no Serbian isolates were represented in the WE, CE or NEE sublineages, whilst the WE variant in particular has been isolated from neighbouring BiH and Montenegro over a significant period of time (1986–2006) remains elusive. This fact may suggest that the fox epizootic that spread to Western Europe, simultaneously moved southwards from Croatia through BiH into Montenegro without contributing to the overall fox rabies epidemiology in Serbia. Topography, in particular the Drina River, may have prevented this variant from entering Serbia from the West. Another hypothesis is that hunting pressure at the territory southwards from the Sava and Danube rivers diminished due to hunting restrictions during the conflicts, which resulted in increased fox density and facilitated rabies spread southwards. This may be supported by the observed decline and then rapid expansion of Balkan RABV population in the Bayesian skyline plots. Alternatively, the WE variant may not have competitively co-existed and the more dominant 'SF' or EE variants persisted, whilst the WE variant became extinct. Retrospective typing may detect single intermediary variants or ancestral variants, which have since been replaced by emerging strains.

The 'FRY dog' isolates identified in cattle in Montenegro (1978) and a dog in Serbia (1971) may be residual representatives of an ancestral dog RABV variant now extinct in the Balkan region, due to the successful elimination of dog-mediated rabies by parenteral vaccination. Dog rabies is

still reported in this region but with the exception of the 1971 isolate RV1146, all of the dog isolates analysed in this study were either EE (in both Serbia and BiH) or WE (in BiH) variants and hence reflect fox-mediated spill-over events rather than dog-mediated rabies.

The nomenclature of European RABV variants (Bourhy *et al.*, 1999) no longer adequately describes their geographical distribution. A review of all European RABV sequences should be undertaken and numerical rather than geographical labels assigned, particularly when neighbouring geographical areas are known to be unstudied. The authors admit that their own nomenclature, e.g. 'SF' given its presence in Romania and Bulgaria, may also be misleading. However, until new variant names are agreed, these problems will challenge all future studies.

Our study contributes to the general understanding that RABV lineages differ in their evolutionary rates depending upon geographical location, population dynamics and selective pressures as shown in the Middle East (David *et al.*, 2007) and in Africa (Hampson *et al.*, 2007; Talbi *et al.*, 2009; Hayman *et al.*, 2011). Such investigations uncover the dynamics and mechanisms of viral evolution and transmission and will contribute to future control programmes. This study highlights the population expansion of sylvatic RABV in Europe and the possible associated effects of 'anthropological interference' on its evolution and spread.

METHODS

Virus isolates. A panel of samples from the Balkans (n=210 original brain material) was obtained from viral archives at Pasteur Institute Novi Sad, Serbia and Friedrich-Loeffler-Institute, Wusterhausen, Germany (Table 2, Supplementary Tables S1 and S2, available in JGV Online).

RNA extraction and RT-PCR. The protocols used followed previously published methods (Heaton *et al.*, 1997). Briefly, total RNA was extracted directly from the brain material using TRIzol (Invitrogen) following the manufacturer's instructions and resuspended in HPLC grade water (Sigma). Each sample was quantified and the RNA concentration adjusted to 1 μg μl^{-1}. For partial N-gene amplification (606 bp), reverse transcription using the pan-lyssavirus primer JW12 (5′-ATGTAACACCYCTACAATG-3′) was performed as described previously (Heaton *et al.*, 1997). Reverse-transcribed samples (cDNA) were diluted 10-fold with H_2O (to a final volume of 100 μl). A volume of 5 μl cDNA was used in conjunction with AmpliTaq Gold polymerase (ABI) and primers JW12 and JW6DPL (5′-CAATTCGCACACATTTTGTG-3′). For full N- and G-gene amplification, reverse transcription was performed with 2 μg of total RNA and 2 pmol of JW12 primer using Superscript III (Invitrogen) in a 20 μl final volume following the manufacturer's instructions. Long distance PCRs were undertaken using Elongase (Invitrogen) following the manufacturer's instructions. To amplify the full N-gene, primers JW12 and 304Rev (5′-TTGACGAAGATCTTGCTCAT-3′) were used, resulting in an amplicon of approximately 1.5 kb. To amplify a 2.7 kb region of the RABV G-gene the primers MF2 (5′-CTATTAACATCCCTCAAAAG-3′) and N-L Rev (5′-TCCCAGTCT-AGGGCRTTCATG-3′) were employed. Amplified products were visualized by agarose gel electrophoresis (2 %) with ethidium bromide. The products were purified using the QIAquick PCR purification kit or gel extraction kit (Qiagen) following the manufacturer's instructions.

Sequencing of PCR products. Purified PCR products were sequenced directly (primers available on request), either using the Big Dye sequencing kit (Applied Biosystems) or the Quickstart sequencing kit (Beckman-Coulter) on the ABI 3100 or Beckman CEQ8800 machines, respectively.

Sequence analysis and phylogenetics. Forward and reverse 400 bp N-gene sequences for each isolate were aligned using Seqman (Lasergene, DNASTAR) and consensus sequences obtained. For ease of analysis, duplicate sequences were removed. Details of the clusters of identical sequences are given in Supplementary Table S2, identified by the group labels I–XXVI (Fig. 2). The remaining unique sequences (n=61) were compared to other previously published RABV sequences (n=94) using a common 400 bp region (Supplementary Table S1). Multiple sequence alignments were performed in CLUSTAL_X using a multiple sequence format file (msf) created in MEGALIGN (Lasergene, DNASTAR). Transition/transversion ratios were calculated in the Puzzle 4.0.2 programme of the PHYLIP package version 3.5 (Felsenstein, 1989). Phylograms were generated using the maximum-likelihood parameter of the DNADIST and neighbour-joining programmes with bootstrap resampling of 1000 replicates as described previously (Johnson *et al.*, 2003).

To investigate the evolutionary history of West Balkan RABV, a panel of 67 RABV N-gene sequences (N1353 nucleotide), collected between 1972 and 2005 from a range of species were analysed, including available published sequences from GenBank (Supplementary Table S1).

To determine the optimal region for evolutionary analysis, a subset of 37 West Balkan RABV, collected between 1972 and 2000, were analysed for which both G-gene and N-gene data were available. Five datasets were prepared representing partial N-gene (N400), complete

Table 3. Mean TMRCAs and nucleotide substitution rates per site per year calculated from duplicate BEAST analyses (uncorrelated lognormal relaxed clock, SRD06 model, coalescent constant size tree prior) for 37 FRY RABV using five datasets: N (N400; N1353 nt), G (G600, G1402) and concatenated (NG2755)

Size (bp)	Divergence time (MRCA)			Substitution rate (substitutions per site per year)		
	95 % HPD upper	95 % HPD lower	Mean	95 % HPD upper	95 % HPD lower	Mean
N400	1779	1932	1862	4.60×10^{-4}	1.34×10^{-4}	2.89×10^{-4}
N1353	1869	1930	1901	5.21×10^{-4}	2.84×10^{-4}	4.04×10^{-4}
G600	1858	1954	1909	7.02×10^{-4}	2.38×10^{-4}	4.55×10^{-4}
G1402	1815	1933	1877	4.82×10^{-4}	2.02×10^{-4}	3.30×10^{-4}
NG2755	1851	1914	1885	4.28×10^{-4}	2.57×10^{-4}	3.40×10^{-4}

N-gene (N1353), partial G-gene (G600), extended G-gene (G1402) and concatenated N- and G-gene (NG2755).

The MCC phylogenetic tree, estimates of the rate of molecular evolution (substitutions per site per year) and the TMRCA for the alignments were inferred using a Bayesian MCMC method in the BEAST package (BEAST and associated programmes are available via http://beast.bio.ed.ac.uk/) (Drummond & Rambaut, 2007). The HKY (SRD06) model of nucleotide substitution model, incorporating a gamma distribution of rate variation among sites (Γ4) was used for the BEAST analysis. To compare the variability in nucleotide substitution rates, models of both strict and relaxed (uncorrelated exponential and lognormal) molecular clocks were tested. Bayes factor analysis (estimated in the Tracer programme) strongly recommended a relaxed (uncorrelated lognormal) molecular clock approach. The statistical uncertainty in the data for each parameter estimate is reflected by the value of the 95 % HPD.

For this analysis, an input file for BEAST was generated using the BEAUti programme with sequences annotated accordingly (Sample ID_Location_Host_Year Isolated.seq). For each estimate, duplicate BEAST runs were performed to test the reproducibility of the analysis. The BEAST output was assessed using the TRACER programme. For each analysis, a chain length of 15 million steps resulted in an effective sampling size (ESS >200 unless noted), with 10 % burn-in removed. Trees and parameters were recorded every 6000 steps. The trees obtained from BEAST were used as input for the TREEANNOTATOR programme to find the MCC tree. Phylogenetic trees were edited for publication using FigTree (version 1.3.1; http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut, 2007). Posterior probability values represent the degree of support for each node on the tree.

Bayesian skyline plot analyses were conducted as above for 37 Balkan RABV on three datasets N1353, G1402 and concatenated NG2755 using the Coalescent Bayesian Skyline as the tree prior (10 groups and ESS >200) (Drummond *et al.*, 2005).

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