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Delimitation of the tick-borne flaviviruses. Resolving the tick-borne encephalitis virus and louping-ill virus paraphyletic taxa

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ABSTRACT

The tick-borne flavivirus (TBFV) group contains at least 12 members where five of them are important pathogens of humans inducing diseases with varying severity (from mild fever forms to acute encephalitis). The taxonomy structure of TBFV is not fully clarified at present. In particular, there is a number of paraphyletic issues of tick-borne encephalitis virus (TBEV) and louping-ill virus (LIV). In this study, we aimed to apply different bioinformatic approaches to analyze all available complete genome amino acid sequences to delineate TBFV members at the species level. Results showed that the European subtype of TBEV (TBEV-E) is a distinct species unit. LIV, in turn, should be separated into two species. Additional analysis of TBEV and LIV antigenic determinant diversity also demonstrate that TBEV-E and LIV are significantly different both from each other and from the other TBEV subtypes. The analysis of available literature provided data on other virus phenotypic particularities that supported our hypothesis. So, within the TBEV + LIV paraphyletic group, we offer to assign four species to get a more accurate understanding of the TBFV interspecies structure according to the modern monophyletic conception.

1. Introduction

As of July 2021, genus *Flavivirus* includes 53 species and more than 40 of them are pathogenic for humans. In accordance with a vector, flaviviruses can be divided into the tick-borne flavivirus (TBFV) group, the mosquito-borne flavivirus group, and the no known vector group (Grard et al., 2007; Moureau et al., 2015).

TBFV are a large group of arboviruses transmitted by hard and soft ticks. Members of the TBFV are widely dispersed across Africa, Europe, Asia, Oceania, and North America (Heinze et al., 2012). TBFV may infect vertebrates which can be reservoirs and play a vital role in maintenance of viruses in natural foci.

The TBFV group has 12 members: Louping ill virus (LIV), Kyasanur Forest diseases virus (KFDV), Powassan virus (POWV), Omsk haemorrhagic fever (OHFV), tick-borne encephalitis virus (TBEV), Gadgets Gully virus (GGYV), Langat virus (LGTV), Royal Farm virus (RFV), Meaban virus (MEAV), Saumarez Reef virus (SREV), Tyuleniy virus (TYUV), Kadam virus (KADV); the first five of them (LIV, KFDV, POWV, OHFV, and TBEV) are important pathogens of humans also known as the "tick-borne encephalitis (TBE) serocomplex": OHFV and KFDV cause haemorrhagic fever in humans, other three viruses (LIV, POWV, and TBEV) induce meningitis, encephalitis, and meningoencephalitis (Shi et al., 2018). The most notorious member of this complex is TBEV. About 12,000 tick-borne encephalitis (TBE) cases are detected annually. Foci of TBEV have been identified in the Russia, Europe, northern China, South Korea, and Japan (Dobler et al., 2017). Recently, Fares et al. (2020) have reported the presence of TBEV (European subtype) in northern Africa (Tunisia).

Not so long ago, taxonomy rearrangements within the TBFV group have taken place. Based on genetic analysis of a polyprotein and an envelope protein of KFDV and Alkhumra (also frequently noticed in the literature as "Alkhurma" – synonymous introduced by typo)

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haemorrhagic fever virus (AHFV), species *Kyasanur Forest diseases virus* and *Alkhumra haemorrhagic fever virus* have been fused into the one taxon – *Kyasanur Forest diseases virus* (Charrel et al., 2001). Also, considering genetic distances, species *Powassan virus* and *Deer tick virus* have been merged into one as well (Beasley et al., 2001).

Interesting taxonomy perturbations have occurred with species *Royal farm virus* and *Karshi virus*: according to the International Committee on Taxonomy of Viruses (ICTV) these two species had been merged in 1999 with no specific reasons mentioned in the available literature. Here, it is important to note that the phylogenetic distance between RFV and Karshi virus (KFV) exceeds empirical interspecies threshold (the most recent common ancestor (MRCA) of RFV and KFV is located approximately in the middle of the tree) regarding the other TBFV species (Grard et al., 2007; Moureau et al., 2015).

Another vague situation in terms of taxonomy is observed within the TBEV group: on the phylogenetic trees, LIV is a sister group of the European subtype of the TBEV clade (Dai et al., 2018; Uzcátegui et al., 2012), thus the species *Tick-borne encephalitis virus* is a paraphyletic group. This fact contradicts not only modern cladistics, but also the ICTV definition of the species taxon: "A species is a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria". In addition to monophyly and genomes relatedness, ICTV also considers the following criteria: natural and experimental host range, cell and tissue tropism, pathogenicity, vector specificity, and antigenicity (https://talk.ictvonline.org/information/w/ictv-information/383/ictv-code). Thus, the taxonomy status of the TBEV + LIV group remains unclear. Also, there are several LIV-like viruses (Spanish goat encephalitis virus (SGEV), Spanish sheep encephalitis virus (SSEV), Turkish sheep encephalitis virus (TSEV), and Greek goat encephalitis virus (GGEV)) that are not currently classified and not included in ICTV master species lists.

The intraspecies structure of TBEV is presented by five main subtypes (listed in the order they were discovered and described): the Far-Eastern (TBEV-FE), the European (TBEV-E), the Siberian (TBEV-S), the Baikalian (TBEV-B) (Adelshin et al., 2019; Demina et al., 2012; Demina et al., 2021; Kozlova et al., 2018; Zlobin and Malov, 2015) and the Himalayan (TBEV-H) (Dai et al., 2018) which are generally demarcated by genetic distances (Deviatkin et al. (2020) recently proposed 10 % nucleotide distance criterion for the ORF gene) and serologically. The subtype names point out their prevalent geographic distribution, however, for TBEV-E and TBEV-S, there are "irregular" isolates found far from their primary foci. On a phylogenetic tree, the TBEV subtypes are all monophyletic groups and divided by internal branches with the lengths possibly being long enough to delineate these subtypes as species taxa.

The final solution on the TBFV taxonomy issue is important concerning epidemiology and prevention. A virus species due to the natural selection obtains specific biological properties allowing them to adopt to specific host range. In the case of TBFV, during infection, primary cell barrier is overcome due to physicochemical interactions between virus envelope glycoprotein (E protein) and receptors on the host cell surface. Amino acid sequences of the E protein of different TBFVs determine their specific host range. In humans, E protein is the main target of immune response both after natural infection and vaccination. Several studies showed substantial variation of the E protein of TBEV subtypes that reduce cross-immune response to infection by different TBEV strains (Bukin et al., 2017; Rey et al., 1995). Clarification of taxonomy status of different TBFV members can aid universal multivalent vaccine developers to improve prevention of virus infections.

This study aimed to clarify the ambiguity in the taxonomy structure of the TBFV group using three molecular species delimitation methods and all available complete genome data. Then, we focused on the analysis of the most dangerous and widespread group of TBEV (including LIV). For TBEV and LIV, we carried out analysis of antigenic determinants of an envelope protein (E) to clarify the issue of interspecies position of them. In conclusion, we analysed available literature on the remaining species criteria to make our approach in determining the interspecific threshold more comprehensive and holistic.

2. Materials and methods

2.1. Genome data set preparation

To delimit species units within the TBFV group, amino acid (aa) sequences of a complete ORF (3414 aa) available in ViPR (Pickett et al., 2012) and NCBI were used. According to the current taxonomy state, for each species, at least one sequence was found. A total of 278 amino acid sequences were used in the analysis (Table 1).

As the whole-genome data is limited, to perform more robust analysis, we gathered the data set consist of the E gene amino acid sequences of TBEV and LIV – the group where paraphyletic issues are most clear. The same data set was used for the comparative analysis of E protein antigenic determinant sequences of TBEV and LIV (see section 2.4.). The TBEV Sofjin strain (1488 nt) was used for a nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for homologous E gene nucleotide sequences for TBEV and LIV in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). BLAST parameters were set as following: word size was set as 11; match/mismatch scores – 2, −3; gap costs – existence: 5, extensions: 2. Initially, 982 nucleotide sequences were found. The nucleotide data set obtained was translated to amino acids and filtered by a length threshold of 367 aa (~75 % of E protein). Using the resulting data set (932 sequences), we performed phylogenetic analysis with IQTREE v.1.6.12 to determine a virus subtype. As a result, we assigned the next five phylogenetic groups: TBEV-FE, TBEV-S, TBEV-E, TBEV-B and LIV. TBEV-H and other TBEV lineages were excluded cause of an insufficient number of sequences for inter- and intragroup statistical analysis. After that, based on the published crystallography results (Rey et al., 1995), fragments exposed at the virus surface – the E protein antigenic determinants, – were defined and isolated from full-length amino acid sequences of the E protein (the length of antigenic determinants was 224 aa, Fig. 1); for more information on this procedure see Bukin et al. (2017)). For the amino acid sequences of antigenic determinants, a length threshold was set as 190 aa (85% of total determinants length). The final alignment comprised 812 antigenic determinant amino acid sequences of TBEV and LIV (Table 2). Sequence alignment, ML tree and delimitation output files can be downloaded from: https://doi.org/10.6084/m9.figshare.17059673.

Sequences were visualised with AliView v.1.26 (Larsson, 2014) and aligned with MAFFT v.7 online (Katoh et al., 2017; Kuraku et al., 2013).

2.2. Phylogenetic analysis and model selection

Phylogenetic analysis was performed with BEAST v.1.10.4 (Suchard et al., 2018) and IQTREE v. 1.6.12 (Nguyen et al., 2015) as implemented

Table 1
The number of amino acid sequences of an ORF region (~3414 aa) for each TBFV group member used for phylogenetic reconstruction and species delimitation.

Member	A number of ORF sequences
GGYV	2
KADV	1
KFDV + AHFV	25
LGTV	3
LIV + LIV-like	30
MEAV	1
OHFV	3
POWV + DTV	23
RFV + KSIV	5
SREV	1
TBEV	181
TYUV	3
Total	**278**

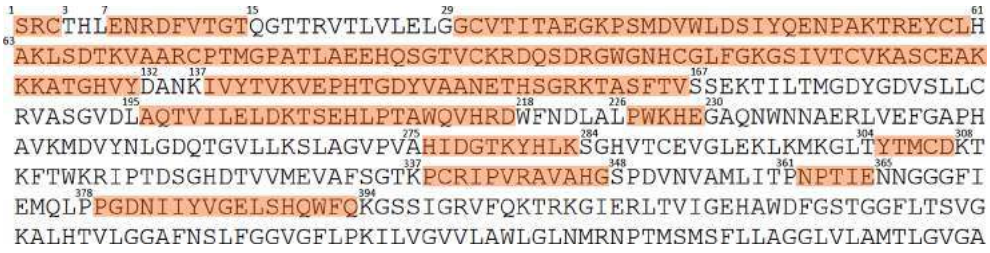

Fig. 1. The scheme of the surface antigenic determinants of the E gene used in the comparative analysis *in silico*.

Table 2
The number of sequences used in antigenic determinants comparative analysis.

Phylogenetic group	Number of sequences
TBEV-FE	293
TBEV-S	159
TBEV-E	308
TBEV-B	12
LIV	40
Total	**812**

on the CIPRES web server (Miller et al., 2010). The best-fit amino acid substitution matrix with the lowest value of the Bayesian information criterion (BIC) (FLU + G_4 + I) was chosen by ModelFinder (Kalyaanamoorthy et al., 2017); number of gamma categories, alpha (shape) parameter of a gamma distribution (0.78) and proportion of invariant sites (0.13) were fixed in further analysis in BEAST. Based on coefficient of variation values of substitution rates (a mean value = 0.58; 95% HPD, 0.49–0.68), the relaxed clock with an uncorrelated lognormal distribution (UCLD) was selected as a molecular clock model. The birth–death (BD) model was chosen over a Yule prior since a preliminary BEAST run has demonstrated that a 95 % highest posterior density (HPD) interval of death rate lay far enough from zero (0.991–0.999).

For the amino acid sequences of the E protein of TBEV and LIV group, phylogenetic analysis was performed analogically as for the complete ORF data set with BEAST and IQTREE programs. For the BEAST analysis, we used substitution matrix FLU + G_4 which has the lowest BIC value for this data set (see supplementary materials), UCLD clocks and the BD speciation model.

In all evolutionary reconstruction, the reproducibility of each Markov chain Monte Carlo (MCMC) analysis was tested by five independent BEAST runs. Each MCMC analyses was run for 100 million iterations, with a tree sampled every 2,500 steps. Burn-in proportion was selected in each run individually. Then, we combined all analysis logs (*.log and *.trees file of the BEAST output) in LogCombiner and analysed the summary log with TreeAnnotator to obtain the most credible clade tree. The convergence and effective sample sizes (ESS) of the summary log were assessed using a Tracer v.1.7.1 program (Rambaut et al., 2018). The BEAST project file, the consensus tree and the output Tracer logs (combined by LogCombiner) are available from figshare.com (https://figshare.com/projects/TBFV_Delimitation/96875).

2.3. Species delimitation

To delineate TBFV species, for the complete ORF aa sequences and TBEV + LIV E protein aa sequences, we employed three bioinformatics delimitation methods. The maximum likelihood tree, reconstructed in IQTREE, was rooted by Apoi virus (NC_003676) as an outgroup and used to delimit species by a Bayesian implementation of the Poisson tree processes (PTP) model (Zhang et al., 2013) using an online service: https://species.h-its.org/.

The generalized mixed Yule coalescent (GMYC) method (Fujisawa and Barraclough, 2013) implemented in the "splits" package for the R was applied to determine clusters at the species level on the ultrametric tree previously reconstructed with BEAST.

The amino acid distance matrix calculated by the maximum likelihood method implemented in IQTREE was used for species delimitation by using the Automatic barcode gap discovery (ABGD) method (Puillandre et al., 2012) with the online service: https://www.abi.snv.jussieu.fr/public/abgd/.

2.4. Comparative analysis of E protein antigenic determinant sequences of TBEV and LIV

To calculate the inter- and intragroup pairwise protein evolutionary distances for antigenic determinants, we performed phylogenetic analysis by IQTREE v.1.6.12 with 1000 ultrafast bootstrap replicates (Hoang et al., 2018) using the E gene amino acid data set (812 sequences). The best-fit amino acid substitution matrix according to the lowest BIC values calculated by ModelFinder was HIVb + G4. The sample of reconstructed trees (bootstrap replicates) was converted into a set of protein evolutionary distance matrices (1000 matrices), which were used to calculate the value of the F_{st} criterion - the measure of intergroup (the groups were TBEV-FE, TBEV-S, TBEV-E, TBEV-B and LIV) subdivision (Hudson et al., 1992) by the formula:

$$F_{st} = 1 - \frac{H_w}{H_b},$$

where H_w are mean intragroup evolutionary distances, H_b are intergroup evolutionary distances. F_{st} value varies between 0 and 1, values close to 0 indicate the absence of intergroup subdivision, values close to 1 - high subdivision. Inter- and intragroup pairwise distances were analysed with 95% confidential intervals (CIs). The p-values were determined as a proportion of negative or zero F_{st} values from distances matrices (1000 matrices in total) obtained from the total number of bootstrap replicates. If p-values $\geq$ 0.05, then there was no intergroup subdivision (two groups of sequences are part of one homogeneous group). Visualization of violine plots was executed in R with the Vioplot v.0.2. package (https://github.com/TomKellyGenetics/vioplot).

All calculations were performed using a custom R programming language script that available from https://figshare.com/articles/software/R_script_for_Inter_and_intragroup_protein_phylogenetic_distances_analysis/14774094.

3. Results

3.1. Phylogenetic analysis

Results of phylogenetic analysis performed in BEAST revealed a clear asymmetric tree shape with very high posterior probability (pp) of main nodes (Supplemental Fig. 1) except for the KADV isolate with pp of 0.46; its phylogenetic position regarding the other TBFV members remains uncertain (Fig. 2). All the species clusters revealed (which are not singletons) have pp of 1.

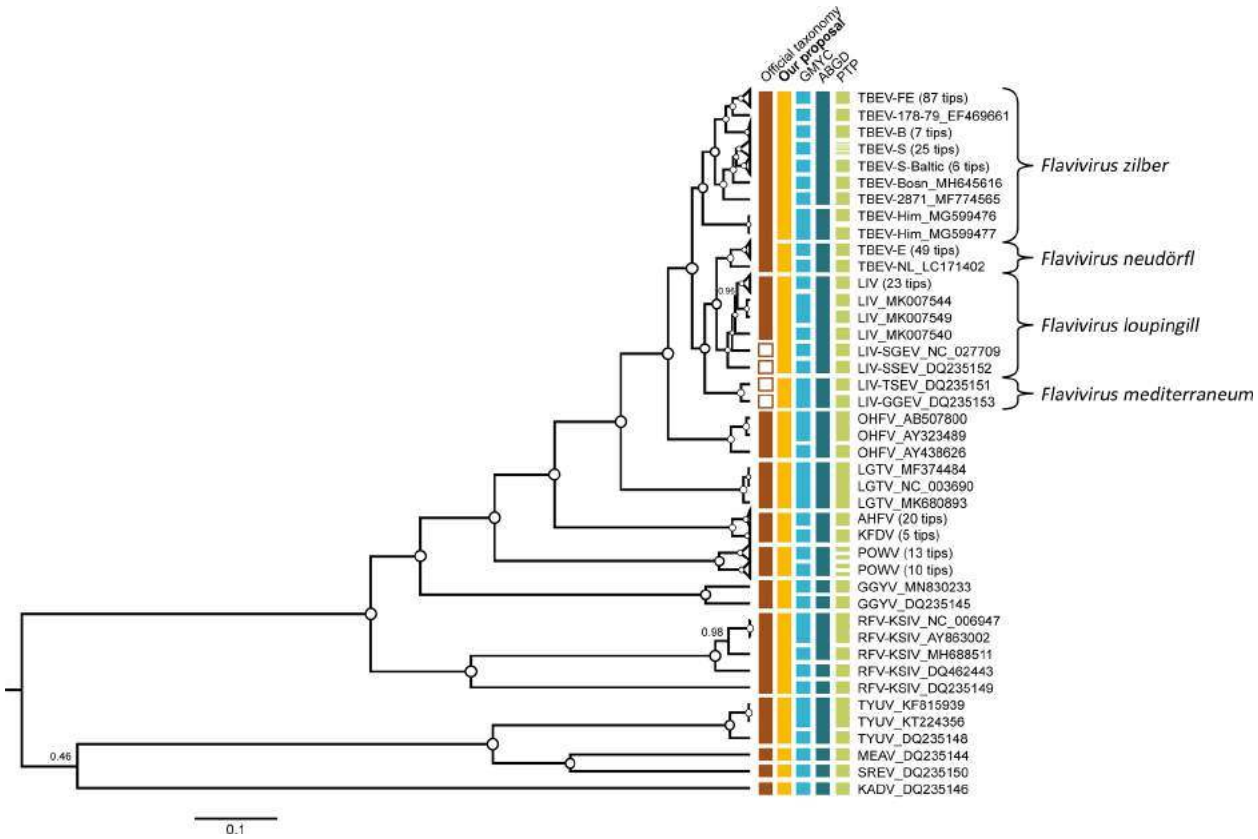

Fig. 2. The phylogenetic tree of the TBFVs. The tree was reconstructed in BEAST using complete amino acid sequences (n = 278) of the polyprotein (3414 aa). For clarity, some of the wide clades were collapsed. Vertical bars to the right of tree tips indicate official classification (brown), our taxonomy proposal (orange), and delimitation results. Internal nodes with pp = 1 are marked as white circles, otherwise support values are shown by numbers ranging from 0 to 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Delimitation results and discrepancies with the official taxonomy

3.2.1. Analysis of complete ORF sequences

The TBFV group was divided into 34, 18, 44 species units by the GMYC, ABGD and PTP analysis, respectively (Table 3).

The discrepancy between the official taxonomy and the delineation results is observed within the TBEV + LIV paraphyletic group and OHFV, KFDV + AHFV, POWV, GGYV, RFV + KSIV, TYUV monophyletic groups (Fig. 2).

The TYUV cluster was divided into two species units according to GMYC and PTP methods. The isolate DQ235148 from the Three Arch Rocks National Wildlife Refuge (USA, Oregon) was separated from two other isolates (KF815939, KT224356) from the Russian Far East (the Sea of Okhotsk, Tyuleny Island), with air distance between these isolation places being about 6,000 km.

The RFV + KSIV isolates from the Central Asia (Uzbekistan, Afghanistan, Turkmenistan, and Northwest China) showed relatively high intragroup amino acid diversity despite their geographic distance is relatively small. Notably that the isolate DQ235149 (Afghanistan) diverged from other RFV + KSIV members more than TUYV diverged from SREV and MEAV. The GMYC, the ABGD and the PTP algorithms split the RFV + KSIV cluster into 4, 3, 4 distinct species units, respectively.

The GGYV cluster formed by two isolates (MN830233, DQ235145) from Australia and Antarctica has split into two species units by all three methods.

The POWV group has split into two species units by the GMYC method, however the PTP algorithm discovered 4 species. The POWV group consists of two distinct clades, which have a clear geographical determinant (the Russian Far-East and USA) of isolates clustering (Supplemental Fig. 2). The PTP method delimits the each of two main cluster into two species units. The GMYC methods identified two main clusters as two species units without splitting them within. ABGD showed the most conservative point of view and did not split POWV cluster as it is in the official taxonomy. Thus, delimitation methods remained POWV taxonomy structure unresolved.

According to GMYC and PTP, the KFDV + AHFV cluster is delimited into two distinct species – KFDV (India) and AHFV (Saudi Arabia). In turn, the ABGD support official taxonomy status of the RFDV + AHFV group as a single species taxon.

The OHFV group was divided into two species units by the GMYC and PTP methods, and the ABGD method defined the cluster as a single species.

Delimitation methods showed the most inconsistency in the case of the TBEV + LIV paraphyletic group. All three delineation methods defined TBEV and LIV as distinct species, however interspecies separation in both viruses was different. Generally, within the TBEV group, the

Table 3
Results of the species delimitation tests in the TBFV group.

Gene	Virus group	Number of sequences	A number of species clusters / interspecies threshold (aa distance)		
			GMYC (p-value for LR-test)	ABGD	PTP*
Polyprotein	TBFV	278	34 (1.3E-9) / 0.0271	18 / 0.0278	44 / -
E	TBEV + LIV	812	40 (0.0) / 0.0179	8 / 0.022	589 / -

* - PTP has floating interspecies threshold.

GMYC and PTP methods, compared to ABGD, delimited species more frequently – 10, 16, and 3 species, respectively. Concerning LIV and LIV-like viruses, ABGD once again was more conservative (2 species clusters – LIV + SGEV + SSEV and TSEV + GGEV), whereas GMYC and PTP determined 6 and 8 species, respectively. Notably, TSEV isolated in Turkey and GGEV isolated in Greece were not only delimited by all of three methods but they were also geographically distant from LIV (British Isles) and both LIV-like viruses (Spain). In this case, phylogenetic analysis provided strong evidence of geographic clustering, which is also consistent with delimitation analysis. Since the TBEV + LIV group is most representative in terms of a number of available sequences and literature information on the other viral species criteria (e.g., pathogenicity, cell and tissue tropism, vector specificity, etc.), we decided to do the additional analysis of this group. We have analysed the envelope protein amino acid sequences of TBEV and LIV to distinguish these putative viruses considering their antigenic properties.

3.2.2. Analysis of E gene sequences

The results of the delimitation analysis of the TBEV + LIV group using 812 E gene sequences showed extremely high sensitivity of the PTP method which detected 589 species entities in compared with 24 species in the case of complete ORF sequences. GMYC, in its turn, revealed 40 species units VS 16 units in the ORF analysis. ABGD was the most stable and detected 8 species within the TBEV + LIV group that is comparable with the complete ORF analysis results (5 species).

3.3. Comparing antigenic determinants of TBEV and LIV

The analysis of protein evolutionary distances between the antigenic determinants of TBEV and LIV showed that LIV is statistically different from all TBEV subtypes (including TBEV-E; Fig. 3, Table 4).

LIV intragroup distances have the highest mean value and the widest 95% CI that, in turn, indicate the highest antigenic polymorphism of LIV (Fig. 4). However, 95% CI of LIV are visibly overlapped with TBEV-FE and TBEV-S CIs.

Table 4
Inter- and intragroup pairwise protein evolutionary distances of TBEV and LIV antigenic determinants.

Comparing virus pairs	Mean intragroup distance	Mean intergroup distance	F_{st}	P-value
S-B	0.0062	0.0115	0.3666	0
FE-B	0.0047	0.0096	0.3968	
FE-S	0.0102	0.0203	0.4424	
*E-S	0.0088	0.0327	**0.7003**	
FE-E	0.0073	0.0410	**0.7961**	
E-LIV	0.0122	0.0817	**0.8424**	
S-LIV	0.0152	0.1080	**0.8541**	
FE-LIV	0.0136	0.1163	**0.8773**	
E-B	0.0033	0.0322	**0.8794**	
B-LIV	0.0096	0.1075	**0.9069**	

* - pairs with TBEV-E or/and LIV were bolded.

As with LIV, TBEV-E are significant different from other TBEV subtypes (95% CI overlap was not shown; Supplemental Fig. 3c, g), with an exception of TBEV-S (there is a slight overlap of 95% CIs; Supplemental Fig. 3e). In contrast, the inter- and intragroup protein evolutionary distances of the remaining three TBEV subtypes (TBEV-FE, TBEV-B, TBEV-S) do not significantly differ (there is overlapping of CIs; Supplemental Fig. 3b, d, f).

In all cases of comparing TBEV-E and LIV versus other TBEV subtypes, F_{st} values were more than 0.5 (Table 4). Notably, in the same table we can see that the mean values of intergroup distances of all viruses analysed are more than intragroup distances, but, as we discussed above, CIs of TBEV-FE, -B and -S pairwise inter- and intragroup distances display overlap and, as a consequence, are not statistically distinct.

On the consensus phylogenetic tree (Supplemental Fig. 4) of TBEV and LIV reconstructed using the amino acid sequences of antigenic determinants, TBEV-S has no reliable bootstrap support (91 and 45 score for ultrafast bootstrap and SH-aLRT methods, respectively) and,

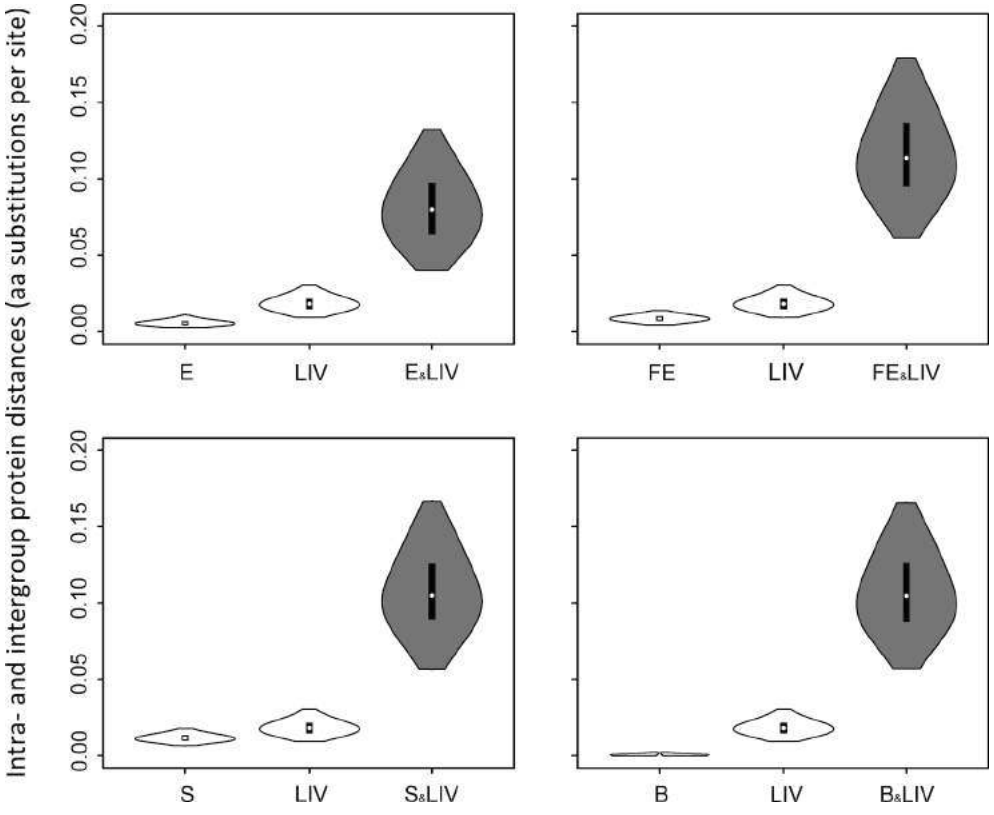

Fig. 3. Comparing intra- and intergroup pairwise protein evolutionary distances of LIV and TBEV epitopes (224 aa). The distances were calculated based on 1000 replicates of ultrafast bootstrap analysis using 812 amino acid sequences. Distributions of intra- and intergroup pairwise distances are displayed as white and grey violin plots, respectively. The upper and lower boundaries of violin plots represent 95% CIs. Black vertical bars within plots are standard deviation, white circles – a mean value. The Y axis shows protein evolutionary distances expressed in amino acid residue substitutions per site. On the X axis, there are TBEV subtypes (FE – Far-Eastern, S – Siberian, E – European, B – Baikalian) and LIV.

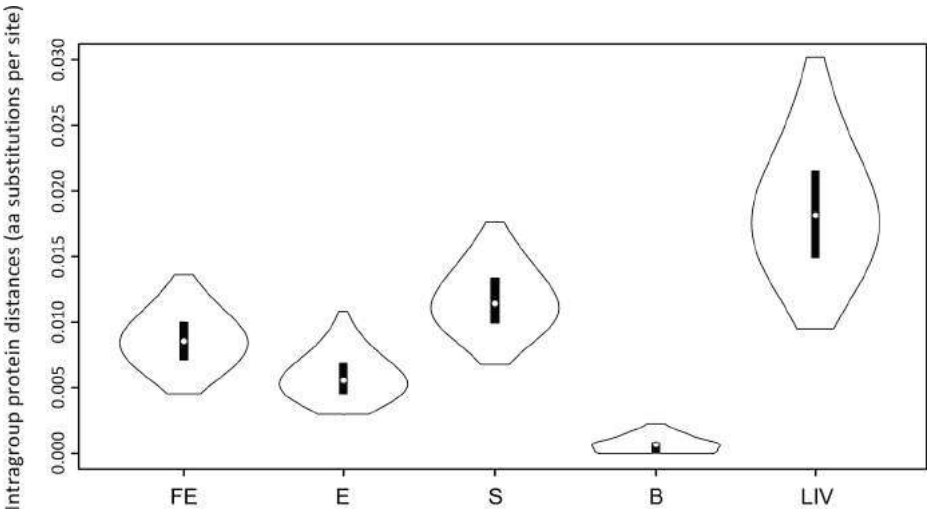

Fig. 4. Distributions of intragroup pairwise protein evolutionary distances of LIV and TBEV antigenic determinants (224 aa). The designations are the same as in the Fig. 2.

therefore, cannot be surely separated from TBEV-FE and -B unlike TBEV-E and LIV which have high enough support score (95/94 and 99/100, respectively).

4. Discussion

4.1. Delimitation of TBEV and LIV phylogenetic groups

The phylogenetic analysis based on the complete ORF amino acid sequences of TBFVs inferred the tree topology with high posterior supports where 70% of nodes has posterior probability > 0.8 (Supplemental Fig. 1). This is especially important for the phylogeny-based delimitation methods such as GMYC and PTP. Another factor that has the potential to distort the tree topology is recombination. The genomes of some strains can be formed during the process of recombination between the genomes of different genotypes and virus species. In our data set, the influence of the recombination process on the formation of strains could potentially bias the conclusions of species delimitation, that is especially important in the case of the TBEV, LIV, SGEV and SSEV monophyletic cluster, the taxonomy of which we offer to revise in this study. A number of works (Bertrand et al., 2012; Dzhioev et al., 2015; Norberg et al., 2013; Yun et al., 2011) showed high statistical support for recombination events only within TBEV-FE, -E and -S subtypes. The possibility of recombination between some strains of TBEV-E and LIV viruses has been expressed (Yun et al., 2011). But the results of these studies are refuted by the reanalysis of the data in the works of Bertrand et al. (2012) and Norberg et al. (2013). Bertrand et al. (2012) declared the possible recombination event between the LIV and SSEV strains on the short genome fragment of 326 bp (≈ 3% of the length of the complete genome). Thus, the recombination that occurs only within the subtypes of TBEV, LIV, SGEV and SSEV and resulting in the possible exchange of small fragments of the genome cannot affect the formation of clusters at inter-subtype level and, as a consequence, not biases the results of species delimitation of the new taxa proposed in our work.

Implying all three delimitation methods using complete ORF sequences separated TBEV-E and LIV into the independent species taxa (Fig. 2). Species delineation based on the E gene sequences with PTP and GMYC methods demonstrated overestimation of a number of species entities (589 and 40, respectively) especially in the case of PTP. The last one delimited species cluster with low bootstrap values (see the ML tree reconstructed with IQTREE in Supplementary materials). ABGD determined the number of species entities (8) comparable with the results of complete ORF sequence analysis (5 species clusters) and segregated the entire TBEV-E cluster into the single species (Supplemental Fig. 5) as well as LIV. In turn, TBEV-E is characterised as a virus group with the lowest genetic diversity. The highest genetic diversity is inherent in TBEV-FE + TBEV-S + TBEV-B + TBEV-H group.

The results of the TBEV and LIV antigenic determinants comparison demonstrated that TBEV-E and LIV are probably different from each other and the remaining TBEV subtypes regarding their antigenic properties. Concurrently, TBEV-FE, -B, and -S subtypes are not statistically distinct by antigenic determinants structure.

To keep monophyly principle, we should whether to combine TBEV-E and LIV as a single species, or assign them as two separate taxa. Thereby, to holistically scrutinised this problem, we have analysed available literature for other viral species criteria. It should be noted that according to all three delimitation methods TBEV-H was delineated as separated species as well, however, there is no data on its biological particularities and we will therefore not consider it as an independent taxon.

4.2. Consideration of the biological and ecological peculiarities of TBEV and LIV and comparing them with species delimitation results

It is reliably known that TBEV infects humans causing severe meningitis, encephalitis, and meningoencephalitis. Contrarily, cases of LIV infections in humans are relatively rare, with signs of acute meningoencephalitis and poliomyelitis being described. The clinical picture for humans infected with LIV is very similar for that produced by TBEV-E: The first phase of disease is characterised by fever (2–11 days) followed by remission (5–6 days) and then the re-emergence of fever and meningoencephalitis lasting 4–10 days, usually with full recovery (Gritsun et al., 2003). Notably, a biphasic course is observed in 74% of TBE patients infected with TBEV-E (Kaiser, 1999), but TBEV-FE and -S infections are predominantly monophasic, only a small reminder demonstrating a biphasic pattern (Mansfield et al., 2009). Also, infections with TBEV-FE often cause an illness with a gradual onset, more severe course, higher rates of severe neurologic sequelae compared to TBEV-E infections (Bogovic and Strle, 2015).

One of the most important peculiarities of neurotropic viruses is their ability to across the blood brain barrier (BBB) and cause encephalitis. LIV induce encephalitis in sheep annually, morbidity and mortality rates ranging from 5 to 60% (Jeffries et al., 2014). In contrast, TBEV seems to show nonvirulence for livestock (there are no reports of mass epizootics in Eurasia) and, apparently, persists in wild rodents asymptomatically. In experiments, it was demonstrated that three TBEV subtypes (TBEV-FE, -E, -S) are able to induce encephalitis in bank voles (*Myodes glareolus*) but causing neuronal death in this natural host in very rare cases

(Tonteri et al., 2013). TBEV RNA was detected in the brain of the infected rodents for up to 109 days post infection (dpi) in the case of all three subtypes. Besides, it was shown that TBEV-FE has distinctive infectious kinetics in bank voles: The long duration of TBEV-FE viremia (25 dpi in 3 animals infected with TBEV-FE VS 14 dpi in 1 animal infected with TBEV-S) may suggest a different transmission pattern as compared to TBEV-E. In the analogous study (Achazi et al., 2011), 12 small rodents (*Microtus arvalis*) were subcutaneously infected by the TBEV-E strain "Hypr". As in the study of Tonteri et al. (2013), TBEV-E RNA was detected in the brain of infected animals for up to 100 days post infection. In 1 animal, viremia was detected up to 50 dpi against 14 dpi as it was shown in the work of Tonteri et al. (2013). Presumably, it is due to the fact that the strain "Hypr" exhibits a significantly more virulent phenotype in the mouse model than the TBEV-E prototype strain Neudoerfl (Wallner et al., 1996).

Goats infected with TBEV-E shed the virus with their milk without showing any symptoms (Balogh et al., 2012).

The experimental subcutaneous infection of cows (n = 3) by the strain "Hypr" (TBEV-E) in the neck area has demonstrated no clinical signs of the disease with an exception of minor temperature rise up to 39.1 and 39.3 °C in the case of two cows on the fifth and sixth days after infection (Grešíková, 1958). It's worth mention that the preliminary serum tests have shown the absence of neutralizing antibodies to tick-borne encephalitis virus from all three cows.

Intriguing results were obtained during experiments with sheep (Votiakov et al., 2002). In the number of studies, sheep received virus solution containing TBEV-FE and TBEV-E subcutaneously and by intracerebral infection (15 and 24 sheep for TBEV-FE and -E experiments on intracerebral infection, respectively). It was shown that subcutaneous infection of sheep as well as infection via ticks with TBEV-E didn't yield transition of BBB induced only meningitis. In the case of intracerebral infectious, TBEV-E showed clear biphasic course with neurological symptoms (anisocoria, ptosis, myelitic paresis, tonic-clonic spasm) and mortality rate only 12,5%. The viral titer in the different parts of brain (cortex, cerebellum, medulla, cervical, lumbar) during the first phase (fever phase) ranged 1.2–2.8 $lgLD_{50}$ (mean = 2.0). In the blood, virus titer averaged 2.5 $lgLD_{50}$ and was higher than in the CNS. The morphological studies of the CNS showed glial nodes took 8.5–21.5% of microscope fields of view (FOV; mean – 14,6%). Neuronophagia took only 5.6–10.0% of FOV (mean – 7.4%). Damage to the neurons occurs only in some animals as a secondary inflammatory effect arising from infection of glial cells. Whereas, in contrast, the intracerebral infection of sheep with TBEV-FE (the strain "198") demonstrated mortality rate of 100%. The course of the disease was monophasic and severe and developed rapidly. Already on days 2–3, signs of focal brain damage simultaneously with fever were rapidly developing in sheep. The viral titer in the CNS was on average 1.3 $lgLD_{50}$ higher (1.2–1.8 $lgLD_{50}$) than in the blood. Primary degenerative CNS disorders prevailed. Neuronophagia took 32.5–42.5% of FOV (mean – 37.5%). Glial nodes ranged widely 34.0–99.0% of FOV (mean – 56.1%). For details, see Supplemental Table 1.

The modern study of immune response to LIV and TBEV-E (subcutaneous infection) in sheep demonstrated the detection of virus specific neutralising antibodies in both cases, but only antibodies against TBEV-E showed the control of infection, whereas LIV progressed to a febrile infection which is followed by neuroinvasion (Mansfield et al., 2016).

Votiakov et al. (1978) also showed that European virus initially did not replicate in or damage neuronal cells even after intracerebral infection. Instead, the primary target of European virus was lymphoid tissue and the virus subsequently appeared in the brains, 6–9 days after inoculation (in cerebellum predominantly) of those animals that developed encephalitis. In turn, Far-Eastern virus directly infected and damaged neurons in the brain, resulting in severe encephalitis. These facts evidently indicate that TBEV-FE is more neurotropic than TBEV-E.

The other important particularities of virus species are vector and geographic distribution. It is interesting that for TBEV and LIV ticks are both a vector and a reservoir. It was found that the main vector for TBEV-FE and TBEV-S is *Ixodes persulcatus*, for TBEV-E and LIV, in turn, – *I. ricinus*. The spatial distribution of TBEV and LIV is based mainly on the habitats of these tick species and reservoir hosts: TBEV-E is predominantly distributed in Central Europe, TBEV-S and TBEV-FE are mostly spread throughout Siberia and the Far East. LIV is primarily found in the British Isles (upland areas of Great Britain and Ireland), with records also from the Russian Far-East (Leonova et al., 2015), Norway and Spain (Jeffries et al., 2014). Not so long ago, it was believed that TBEV is absent in the British Isles, though it has been recently shown the presence of TBEV-E in the East of England (Holding et al., 2020).

Considering reservoir transmission hosts, LIV once again demonstrates an obvious difference from TBEV. Unlike all TBEV subtypes, LIV is primarily found in red grouses and sheep inducing encephalitis and high mortality rate in both (78% in red grouses (Gilbert, 2016), 5–60% in sheep (Jeffries et al., 2014)), not small rodents. Although rodents such as field voles (*Microtus agrestis*), bank voles (*M. glareolus*) and wood mice (*Apodemus sylvaticus*) raised an antibody response to infection, they could not produce a substantial viremia and did not support non-viraemic transmission between co-feeding ticks (Gilbert et al., 2000). Furthermore, red grouses tend not to feed adult *I. ricinus* and is not therefore able to maintain transmission cycle without aid of another host that feeds adult ticks (e.g. deer, so-called "reproduction hosts" (Gilbert, 2016)). This leads to the fact that LIV has patchy spatial distribution with different combinations of reservoir hosts occurring. This is exactly opposite of the TBEV transmission patterns and natural foci structure formed by primarily small rodents.

The dissimilarity of the clinical picture, cell tropism, host range specificity, and pathogenicity of TBEV subtypes and LIV may speculatively be explained by differences in the antigenic determinants structure. Hubálek et al. (1995) employed indirect immunofluorescence test and revealed a clear difference between LIV strains and the TBEV-FE prototype strain "Sofjin". On the UPGMA tree, representing of antigenic relationships of the viruses, LIV strains formed a common cluster with a TBEV-E strain, the TBEV-FE strain laying far from them as an outgroup. It is consistent with our results of TBEV and LIV antigenic determinants comparative analysis (Fig. 3).

The data reviewed supports the hypothesis of considering TBEV-E and LIV as distinct virus species.

4.3. Efficacy of the vaccines against different TBEV subtypes

Despite the comparison of antigenic determinants of different TBEV subtypes revealed a significant difference between TBEV-E and TBEV-FE (Fig. 3), the number of studies on the immunogenicity of the western vaccines (FSME-Immun and Encepur) based on the TBEV-E strains ("Neudoerfl" and "K23") have demonstrated the high level of neutralising antibodies (NAb) against different TBEV-FE and TBEV-S strains (Domnich et al., 2014). However, in the recent work, Tuchynskaya et al. (2021) infected inbred white mice (BALB/c) with the strains "Sofjin KGG" (TBEV-FE) and "Vasilchenko" (TBEV-S), whereas the mice were previously vaccinated by two doses of Tick-E-Vac (the strain "Sofjin", TBEV-FE). After the first vaccine dose, seroconversion against the strain "Sofjin KGG" was 100% (with logNAb > 1) and only about 5% against the TBEV-S "Vasilchenko" strain with no animals having measurable NAb titers. After the second vaccine dose, seroconversion against the TBEV-S strain "Vasilchenko" reached almost 100%, logNAb titers exceeded 1 value but they still were significantly lower than it was shown for the TBEV-FE "Sofjin KGG" strain. Moreover, NAb titers against two TBEV-S strains ("Vasilchenko" and "EK-328") significantly differed which indicates the role of intra-subtype antigenic variety in the immune response. Our comparison of antigenic determinants *in silico* did not reveal differences between TBEV-FE and TBEV-S which can be explained by the low sensitivity of an aa distance matrix. Though, as it was shown previously (Bukin et al., 2017), taking into account physicochemical properties of aa residues has revealed the distinction of three

TBEV subtypes (TBEV-FE, -E and -S) that coincide with results of Tuchynskaya et al. (2021).

4.4. Delimitation of the remaining members of the TBFV group

The delimitation methods elucidated cryptic species within following clades: TYUV, RFV + KSIV, GGYV, POWV, KFDV + AHFV, and OHFV. In all of these cases, phylogenetic species concept (members descend from a common ancestor) is kept. Some of the clades (e.g., RFV + KSIV, GGYV) contain distances that obviously exceed the interspecies threshold. In some cases, the situation with cryptic species still remains uncertain.

4.5. Previous taxonomic proposals

Our results conflict with several previous taxonomic proposals. So, Charrel et al. (2001) came to conclusions that TBEV and LIV is a single species based only on the analysis of aa distances of partial genome region (E gene), and, in addition, the number of sequences in their study was relatively small (4 for TBEV and 9 for LIV). Grard et al. (2007) also analysed aa distances but using complete ORF aa sequences with a number of sequences restricted (4 for TBEV and 4 for the LIV-complex (LIV + SSEV + TSEV + GGEV)). Authors proposed the cut-off distance for species demarcation at 0.09 aa (p-distance). None of species delimitation methods was available at that time, therefore, the threshold offered was not methodologically validated. Importantly, both Charrel et al. (2001) and Grard et al. (2007) did not consider other biological particularities for the fusion of TBEV + LIV as required by the modern ICTV rules. Such species demarcation approach considering only genetic distances seems to be one-sided and does not meet the modern requirements of the ICTV.

4.6. Our taxonomy proposal

Taking into account all the phenotypic manifestations of viruses described above as well as our analysis results, we offer to delineate TBEV-E (with the NL lineage so far) and LIV + SGEV + SSEV from the joint TBEV clad into two distinct taxa and assign them as *Flavivirus neudoerfl* (from the TBEV-E prototype strain "Neudoerfl") and *Flavivirus loupingill*, respectively. To keep the conception of monophyly, we propose to join SGEV and SSEV with the LIV clade into a single species. As a consequence, to maintain monophyly, TSEV and GGEV should be considered as a distinct species as well and to be assigned as *Flavivirus mediterraneum*. The other TBEV subtypes (TBEV-FE, TBEV-B, TBEV-S, TBEV-H) being a monophyletic group are treated by us as a single species which we propose assign as *Flavivirus zilber* (from Lev A. Zilber, the leader of the Soviet expedition to the Far East, which resulted in the discovery of TBEV in 1937 (Zlobin et al., 2017); Table 5).

The consideration of taxonomic status of the other virus species outside the TBEV complex is beyond the scope of this study, however our delimitation results indicate possible fields of future TBFV taxonomy investigations.

5. Conclusion

To summaries, we have put the data on all TBEV and LIV particularities observed into a Supplemental Table 3.

In our analysis, all three delimitation methods showed that LIV and TBEV-E are distinct species. The comparison of envelope protein evolutionary distances *in silico* elucidated that LIV and TBEV-E are significantly different from all TBEV subtypes (and from each other, Fig. 3). LIV has shown clear differences in severity of the disease in humans and sheep being biphasic like TBEV-E. LIV also has relatively low CFR (there is only one officially recorded case). TBEV-E as well as LIV has a biphasic course in humans, less disease severity with meningitis prevailing, and shares with LIV the common vector – *I. ricinus*. Also, experiments with animals (such as sheep, goats and cows) showed that TBEV-E cannot cross BBB and did not cause encephalitis and death after subcutaneous infection or infection via ticks.

We believe that the differences described above are sufficient to delineate TBEV-E and LIV (+SSEV and SGEV) from the joint TBEV clade into two distinct species – *Flavivirus neudoerfl* and *Flavivirus loupingill*, respectively. The rest TBEV subtypes (TBEV-FE, -S, -B, H) we proposed to classify as the species *Flavivirus zilber*. TSEV and GGEV can be combined into the single species taxon – *Flavivirus mediterraneum*.

Table 5
Proposal of species names.

Phylogenetic cluster	Current species name	Species name proposed	Clarification
TBEV-FE	*Tick-borne encephalitis virus*	*Flavivirus zilber*	Lev A. Zilber – the leader of the Soviet expedition to the Far East, which resulted in the discovery of TBEV in 1937
TBEV-S	*Tick-borne encephalitis virus*		
TBEV-B	*Tick-borne encephalitis virus*		
TBEV-H	*Tick-borne encephalitis virus*		
TBEV-E	*Tick-borne encephalitis virus*	*Flavivirus neudoerfl*	Neudoerf – the prototype strain name of TBEV-E
LIV	*Louping ill virus*	*Flavivirus loupingill*	The old species name in the binomial format
SGEV	Unclassified		
SSEV	Unclassified		
GGEV	Unclassified	*Flavivirus mediterraneum*	Mediterranean as the virus isolation territory
TSEV	Unclassified		

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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