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Original article

Geographic dispersal and genetic diversity of tick-borne phleboviruses (*Phenuiviridae*, *Phlebovirus*) as revealed by the analysis of L segment sequences

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ABSTRACT

The large diversity of new tick-borne phleboviruses, and the negative impacts of the virulent viruses on human/animal health have led to a growing interest in their analysis. In this report, new insights are brought out into the diversity of putative phleboviruses circulating in Portugal (both the continental territory and the islands of São Miguel, in the Azores, and Madeira), as well as in the Spanish western regions of Extremadura and Castilla and León. Phlebovirus sequences were frequently detected (L-segment) from both questing and feeding ticks, but especially in *Rhipicephalus sanguineus* sensu lato (s.l.) specimens. These sequences were detected in adult ticks, as well as nymphs and eggs, supporting the hypothesis of viral maintenance by vertical transmission. Though multiple genetic groups could be identified in phylogenetic trees (AnLuc, KarMa, RiPar virus 1, and Spanish group 1 and 2), all the sequences from Portugal and Spain shared common ancestry with other viral sequence obtained from samples collected over a large geographic coverage. Spatiotemporal analysis placed Middle-East as the geographic origin of the most recent common ancestor (MRCA) of all phleboviruses analysed in the present study. More recent viral transitions might include migrations from Spain to continental Portugal, and from there to the Portuguese Islands. Our findings suggest that the time of the MRCA of phleboviruses was dated around 225 years ago [95% HPD: 124–387 year before the last sampling date].

1. Introduction

Among the groups of animals that have been the focus of recent viral surveys, invertebrates, and especially mosquitoes and ticks, seem to harbor an unexpected profusion of novel viruses (Shi et al., 2016). Given the overwhelming genetic diversity of the virosphere, not surprisingly many of the mosquito virologic surveys have revealed a myriad of viruses displaying different degrees of virulence towards humans/other animals, or even those with restricted replication in arthropod-cells (Calisher and Higgs, 2018; Junglen and Drosten, 2013; Wilder-Smith et al., 2017). By comparison, less is known about the diversity of viruses from ticks, but recent studies based on metagenomic/next generation sequerncing (NGS) have repeatedly revealed, for example, new phleboviruses among those that comprise the viromes of different ticks (Tokarz et al., 2014; Xia et al., 2015). Interestingly, in addition to simply revealing new putative viruses, this plethora of

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sequence data may also be analysed in a Bayesian phylogeographic inference context in order to track virus geographic spread in space and time, if discrete/continuous location and heterochronus data is available (Baele et al., 2018).

Over time, different studies carried out in our laboratory allowed the identification, and genetic characterization, of different groups of viruses associated with both mosquitoes and ticks collected in Portugal (Carapeta et al., 2015; Esteves et al., 2005; Ferreira et al., 2013; Parreira et al., 2012; Pereira et al., 2017). Very recently, one of these studies, based on the use of pan-phlebovirus primers, revealed somewhat surprising results, as many phlebovirus sequences were found in three different genera of ticks, and a wide range of genetic variability was identified in a conserved section of the viral genome, encoding the viral RNA-dependent RNA polymerase (Pereira et al., 2017).

Since the above mentioned tick-borne virus survey had been restricted, for logistic reasons, to the analysis of ticks collected exclusively in the south of Portugal, in this report we expand on that analysis. For that, we surveyed the presence of phlebovirus sequences in ticks collected all over Portugal, including the Atlantic islands of São-Miguel (Azores) and Madeira. Taking advantage of an institutional collaboration, this survey was also extended to western Spain, where tick sampling was also carried out in the regions of Extremadura and Castilla-León. Furthermore, not only were these viruses sampled over a larger geographic surface, but also the generated sequence data were explored in combination with a phylogeographic approach, in order to assess their usefulness to allow the investigation of the dispersal of some of these viral sequences across space and time.

2. Materials and methods

2.1. Study area and tick collection

This study describes the analysis of putative phlebovirus sequences (L segment) detected in ticks collected between the months of February 2016 and June 2017, at sites distributed in continental Portugal, western Spain, and the islands of São-Miguel and Madeira (see Fig. 1). Altogether, 1220 feeding ticks were collected directly (mostly) from domestic cows (*Bos taurus*), sheep (*Ovis aries*), wild boar (*Sus scrofa*), domestic pig (*Sus scrofa domesticus*) and dogs (*Canis familiaris*), or from the vegetation (773 questing ticks), in the latter case by flagging with a 1 × 1 m cloth over low and high vegetation (Supplementary Table 1a and 1b). All the ticks were kept alive until they were identified at the species level using taxonomic keys (Estrada-Peña et al., 2004; Pérez-Eid, 2006), and then stored in vials at −80 °C until further use. Alternatively, in those situations when immediate transport to the laboratory could not be ensured, the collected specimens were kept up to one week at −20 °C in *RNAlater*® (Ambion, EUA). All the animals were alive at the time ticks were collected from them, which was carried out in compliance with the Portuguese and Spanish legislation for the protection of animals (Decree-Laws 113/2013/Portugal and 53/2013/Spain). All the ticks collected from animals were either kindly provided by either pet owners (in the case of dogs), or by veterinary physicians who have maintained a long-standing collaboration with both IHMT and the University of Extremadura. Therefore, the feeding-ticks used in this study corresponded to a convenience sample, and were collected (i) while the animals were being deparasitized, (ii) when they were being examined for clinical reasons, or (iii) when they were immobilized for vaccination campaigns.

Despite the fact that, in this study, feeding ticks were only collected from the three species of animals mentioned above, the analysis of sequence data also included those collected from a wider range of hosts (as listed in Supplementary Table 2).

2.2. Preparation of tick homogenates, RNA extraction and DNA sequencing

The preparation of tick homogenates (usually in pools of specimens grouped according to species, developmental stage, sex, and geographic origin) was carried out by mechanical disruption in 1.5 ml Eppendorf test tubes using a sterile pestle and 1 ml of NZYzol® (NZYTech, Portugal), followed by their clarification by centrifugation. RNA was extracted from 200 µl of clarified tick homogenate using NZYol®, as indicated by the supplier. The extracted RNA, once dissolved in 30 µl nuclease-free water, served as template for the synthesis of cDNA that was carried out with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) using random hexamers. Detection of phlebovirus sequences (L segment) was performed by PCR using NZYTaq 2X Green Master Mix (NZYTech, Portugal), and the reaction conditions previously described by Matsuno et al. (2015) and a mixture of the ppL1/ppL2 primers (40 pmol each in a multiplex-PCR reaction) as previously described by Pereira et al. (2017). The obtained amplicons (approximately 500 bp) were purified and directly sequenced by Big Dye (Life Technologies, USA) incorporation, resolved in an ABI 3130X, or cloned in pGEMT-easy® (Promega, USA), followed by DNA sequencing of individually purified plasmid DNA molecules. The attempted detection of S-segment sequences was carried out using previously described primers and reaction conditions (Lambert and Lanciotti, 2009).

2.3. Dataset compilation and Bayesian phylodynamics analyses

For the analysis of the largest sequence dataset (including 214 sequences), multiple alignments of nucleotide (nt) sequences were performed using the iterative G-INS-I method as implemented in MAFFT vs. 7 (Katoh and Standley, 2013) followed by their edition using GBlocks (Castresana, 2000). Phylogenetic trees were constructed using both a Maximum Likelihood (ML) and a Bayesian approach, and the best fitting evolutionary model (GTR + Γ + I; GTR-General Time Reversal, Γ-Gamma distribution, I-proportion of invariant sites), as suggested by JModeltest2 (Darriba et al., 2012). Phylogenetic reconstructions based on the ML optimization criterion were carried out using Mega 6.0 software (Tamura et al., 2013), and the stability of the obtained tree topologies assessed by bootstrapping with 1000 re-samplings of the original sequence data. For this dataset phylogenetic reconstruction was also carried out following a Bayesian approach, using MrBayes v3.0b4 (Huelsenbeck et al., 2001). Theses analyses consisted of 1×10^8 generations starting from a random tree and four Markov chains with default heating values, sampled every 5000th generation. Two separate runs were conducted for each analysis, and the first 10% sampled trees discarded as burn-in, before obtaining a consensus tree.

Additionally, a smaller sequence dataset comprising 95 *Phlebovirus* L sequences was analysed. The multiple sequence alignment of these sequences was obtained using MUSCLE (Multiple Sequence Comparison by Log-Expectation) (Edgar, 2004), followed by its manual edition. The final alignment consisted of 165 codons that corresponded to the 495 nt covering part of the RNA polymerase coding sequence. The coalescent process was used to estimate the spatiotemporal scale of the evolutionary process from the dataset, using the GTR + I + Γ nucleotide substitution model, a relaxed uncorrelated lognormal molecular clock model (Ho et al., 2005), a Bayesian skyline plot population growth model and a non-reversible continuous phylogeography model (Lemey et al., 2009). Two independent Markov chain Monte-Carlo (MCMC) runs were performed using BEASTv1.7.5 (Drummond et al., 2012) until 1×10^8 states were sampled, 10% of which were discarded as burn-in. The Tracer software (http://beast.bio.ed.ac.uk/tracer) was used check for convergence and adequate effective sample size (ESS) higher than 150 after a burn-in of 10%. The trees were logged on every 10,000th MCMC step, and the tree sample was summarized using TreeAnnotator software v1.8.3 as a maximum clade credibility (MCC) tree, using median heights as the node heights in the tree. The FigTree v1.4.2 software was used to visualize the phylogenetic trees (http://tree.bio.ed.ac.uk/software/figtree/). The spatiotemporal reconstruction of *Phlebovirus* spread was visualized on Spatial Phylogenetic Reconstruction of Evolutionary Dynamics software - SpreaD3 (Bielejec et al.,

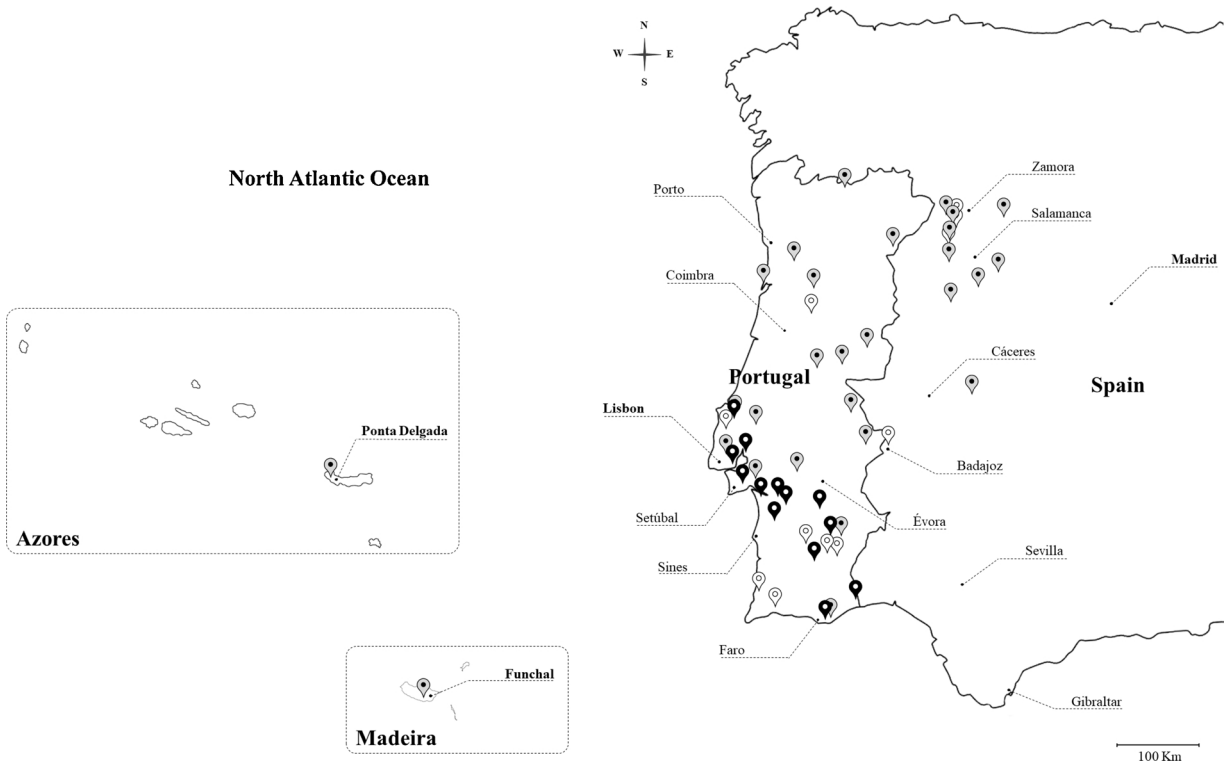

Fig. 1. Distribution over the Portuguese administrative territory and western Spain of the tick collection sites from which phlebovirus sequences were obtained. Grey and black marks represent locations sampled in the course of this study or that carried out by Pereira et al. (2017) and for which virus sequences were obtained. Locations where ticks were collected but for which no Phlebovirus-specific amplification results were obtained are indicated by the white marks. The indicated scale does not apply to the distance of the Azores and Madeira Archipelagoes to continental Europe (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2016), using a custom made geoJSON world map (https://geojson-maps.ash.ms/).

The nt sequences obtained in the course of this study were deposited in the GenBank/EMBL/DDBJ databases under accession numbers LC374218-LC374286 (Supplementary Table 2). The reference sequences used in the analyses presented here were directly downloaded from the public sequence databases.

3. Results

Altogether, 1993 ticks were collected (Supplementary Table 1a and 1b), the majority (n = 1220, 61.2% of the total) corresponding to ticks feeding on hosts including cows, sheep, wild boar, domestic pig, birds and dogs. These encompassed an approximately similar numbers of male and female adult specimens (43.5% and 41.1%, respectively), as well as immature forms (1 larva/0.1% and 186 nymphs/15.2%). The two most abundantly represented species, comprising 77.6% of the specimens collected on animals, corresponded to *Rhipicephalus sanguineus* s.l. (49.9%) and *Hyalomma marginatum* (27.7%). Additionally, questing ticks (n = 773 corresponding to 38.8% of the specimens collected) were sampled from the vegetation, the majority (85.5%) comprising *Ixodes ricinus* (65.8%) and *R. sanguineus* s.l. (19.7%) ticks. Again, male and female specimens were collected in approximate similar numbers (15.4% and 16.4% of the questing tick collection). However, the majority of the collection corresponded to immature specimens, distributed between larvae (51.2%) and nymphs (16.9%).

While the great majority of these ticks were analysed in pools, the total number of specimens still could not be processed. Therefore, we chose to study a smaller sample corresponding to a random selection of 92 pools comprising 746 specimens collected from animals (80.4% of the pools) or from the vegetation (19.6% of the pools). These specimens included both male and female adults (34.7% and 35.8%, respectively), larvae (13.4%) and nymphs (16.1%). The majority of these specimens (89.4%) corresponded to *R. sanguineus* s.l. (49.3%), *I. ricinus* (17.7%), *H. marginatum* (13.1%) and *H. lusitanicum* (9.3%) ticks. Egg postures (n = 3) of *R. sanguineus* s.l. were also analysed (Supplementary Table 2, and Supplementary table 3).

The detection of *Phlebovirus* genomes, we used a one-step multiplex-RT-PCR protocol that explored the use of degenerate primers targeting part of the RNA polymerase coding segment, and this strategy revealed the presence of an amplicon with the expected size in 48.9% (n = 45) of the samples analysed. These included both male and female adult ticks collected either from the vegetation (positive amplification results were observed for 44.4% of the pools), or animals (specific amplicons were observed for 50.0% of the pools). Positive amplification results were very frequently, though not exclusively, associated with *R. sanguineus* s.l. specimens (88.9%), though positive amplification results were also associated with *R. bursa* (6.7%), *H. lusitanicum* and *H. marginatum* (2.2% each). No viral sequences were detected in pools of *Ixodes*, *Dermacentor*, or *Haemaphysalis* ticks, despite the fact that both *Dermacentor* and *Haemaphysalis* ticks have been previously described to harbor these viruses (Li et al., 2015; Papa et al., 2017; Pereira et al., 2017). These results have been summarized in Supplementary Table 3.

As an attempt to further extend the characterization of the detected putative viral genomes, the sequences of the S segment were also targeted for amplification. This was tentatively carried out in a randomly selected group of samples (n = 16) where the presence of an L segment was demonstrated. Previously described primers and reaction conditions (Lambert and Lanciotti, 2009) were used but no amplification results could be obtained.

A total of 691 segment-specific amplicons were sequenced (listed in Supplementary Table 2) (i) either directly or (ii) after cloning in a plasmid vector, followed by DNA sequencing of independent recombinant plasmids (from 4 to 8 per cloning experiment). These

sequences were obtained from ticks collected all over the geographic coverage mentioned above, which is schematically represented in Fig. 1, where the locations of the ticks from which the sequences previously analysed by Pereira et al. (2017) are also indicated. As expected, the initial analysis of these sequences, carried out with MegaBlast, revealed high identity (> 98% over > 95% query coverage, and very low E-values) with homologues previously deposited in the public sequence databases (GenBank/EMBL/DDBJ).

For further characterization of these sequences, phylogenetic analyses were carried out, and these involved the construction of consensus trees using ML and Bayesian approaches, and different datasets. The first, and largest of the assemblages, included 214 sequences (Supplementary Fig. 1) encompassing all those determined in the course of this study, as well as homologous sequences identified by MegaBlast matches, and references representing previously defined *Phlebovirus* genetic lineages (Matsuno et al., 2015). These covered *bona fide* tick- and mosquito/sandfly-borne phleboviruses, as well as viral sequences disclosed as a results of viral screenings carried out using either degenerate primers or metagenomics approaches/NGS (Li et al., 2015; Matsuno et al., 2015; Tokarz et al., 2014).

This initial analysis, clearly placed all the phlebovirus sequences reported here, as well as those previously reported (Pereira et al., 2017), in a single monophyletic cluster of tick-borne phleboviruses supported by both high bootstrap and posterior probability values (collapsed and indicated by the arrow in Supplementary Fig. 1). This cluster also grouped many other viral sequence references obtained from ticks collected in France, Greece, Ghana, Zambia, China, and the USA. However, it excluded the majority of those defining the Uukuniemi, Bhanja and Severe fever with thrombocytopenia syndrome/Heartland groups, as well as a large assembly pf mosquito/sandfly-borne phleboviruses (Matsuno et al., 2015). To obtain a better resolution of the phylogenetic relationships shared by these sequences, a second phylogenetic tree was constructed with a total number of 95 sequences, sampled over a wide geographic range (Supplementary Table 2). This phylogenetic tree (Fig. 2) was constructed using a Bayesian framework and a continuous phylogeographic approach, allowing the assessment of the evolutionary relationships shared between these viral sequences also as a function of time and geographic location.

The three genetic groups of *Phlebovirus* L sequences previously described in southern Portugal (Pereira et al., 2017) could again be identified. The largest of these, designated the AnLuc group, remained monophyletic despite increasing in size, which included the addition of 2 seqs (KX964667 and KX964668) amplified from *Dermacentor* sp. collected in France (Prinz et al., 2017). The KarMa group (also enlarged) was shown to harbor the majority of sequences amplified from *R. sanguineus* s.l. but also from *H. marginatum* (accession number LC146411). One of the most divergent of these sequences, represented by RiPar virus 1 (accession number LC146427), was previously amplified from a pool of questing *D. marginatus* ticks, and shared a common origin with Tacheng tick virus 2 (accession number KM817684; Li et al., 2015). Additionally, two other new viral groups of Spanish origin were identified. These included sequences amplified from *R. sanguineus* s.l. (Spain group 1) and *H. lusitanicum* (Spain group 2), and combined the multiple sequences obtained from independent plasmid clones generated from cloning of two independent amplification products. As expected, sequences derived from independent cloning experiments usually clustered together in monophyletic clusters. However, in one case, a single sequence obtained after analyzing independent plasmid clones from a specific amplicon, revealed one of them (indicated by #, accession number LC374285) to cluster separately from all the others, which formed a monophyletic group (indicated by # in Fig. 2).

Finally, each of the identified clusters of sequences was also characterized by the presence of group-specific combinations of certain amino acid residues in the primary sequence of the L protein they encode (Supplementary Fig. 2).

The phylogeographic analysis here shown placed the approximate origin of the most recent common ancestor (MRCA) of the analysed 95 sequences in the Middle-East (estimated ancestral location is Iraq) little over 225 years ago (Fig. 2). The dispersal of viral sequences in Europe was suggested to have followed different routes (Fig. 3), though most of them seemed to originate from a common origin placed in northern Africa (Libya) little over 85 years ago. However, even the origin of some of the most divergent lineages (RiPar virus 1 and Spain group 2) seemed to coincide with a Middle-Eastern origin, dispersing from there between 90 and 120 years ago (approximately). While the KarMa group and Spain lineage 1 seemed to have diverged from a common ancestor from northern Africa (Algeria), the origin of the MRCA of the AnLuc group seems to have been Spain.

4. Discussion

In this study, a modified version of a previously described one-step pan-phlebovirus amplification protocol (Matsuno et al., 2015) was explored so as to extend the characterization of putative phleboviruses previously identified in ticks from southern Portugal (Pereira et al., 2017). This work had shown that regardless of sex (male/female), origin (questing/feeding) or even species, a surprisingly high number of pools of ticks analysed revealed the presence of viral sequences, which phylogenetic analysis has placed in three different groups in phylogenetic trees.

Given the limited geographic coverage of the tick collections analysed in the above mentioned work, which only included specimens collected in southern Portugal, here we expand on that analysis, and describe the detection and analysis of *Phlebovirus* L sequences in ticks collected all over Portugal (including the Atlantic islands of São-Miguel in the Azores and the island of Madeira), as well as in western Spain, in the regions of Extremadura and Castilla-Léon.

Most of the ticks analysed were combined in pools, and the detection of phlebovirus sequences was indicated by (i) the successful amplification of a specific 500 bp section of the genomic L segment, (ii) their unambiguous match with homologues in the sequence databases, and (iii) their clear association in phylogenetic trees with others phlebovirus sequences. The great majority of the successful amplification results were associated with *Rhipicephalus* ticks, and especially with *R. sanguineus* s.l., but this apparent high infection rate may be impacted by a sampling bias, as this species also comprised the great majority of the specimens collected from animals or the vegetation. The detection of *Phlebovirus*-specific sequences was also successful in most of the tick development stages, including eggs, nymphs and adults. No viral sequences were detected in larvae, but only one pool was analysed.

The amplification of viral sequences from eggs, nymphs and adults (both male and female) supports the hypothesis that these viruses might explore vertical transmission for their maintenance in ticks. However, the detection of viral sequences in eggs did not involve their prior surface sterilization. For this reason, we cannot formally distinguish between viruses on the surface of the eggs (transovum transmission) and those found within the eggs (transovarian transmission). In the first of these situations, the emerging larvae will not necessarily be infected with viruses as previously demonstrated (Chen et al., 2006; Ravoet et al., 2015). Consequently, despite the detection of phlebovirus sequences in almost all the tick developmental stages, a formal proof of vertical transmission of these viruses awaits their detection in larvae hatched from infected eggs. In fact, it is also formally possible to suggest that at least some of the phleboviruses may have been acquired by tick larvae while feeding on a vertebrate host, and then transstadially survived, until they were detected in adult specimens.

Phylogenetic analyses indicated that the viral sequences described in this report segregate into different viral groups, adding two others (Spain lineage 1 and 2) to those previously described (Pereira et al., 2017). Despite the genetic variability found among these sequences, they share a common ancestry. This is indicated by clear clustering in a

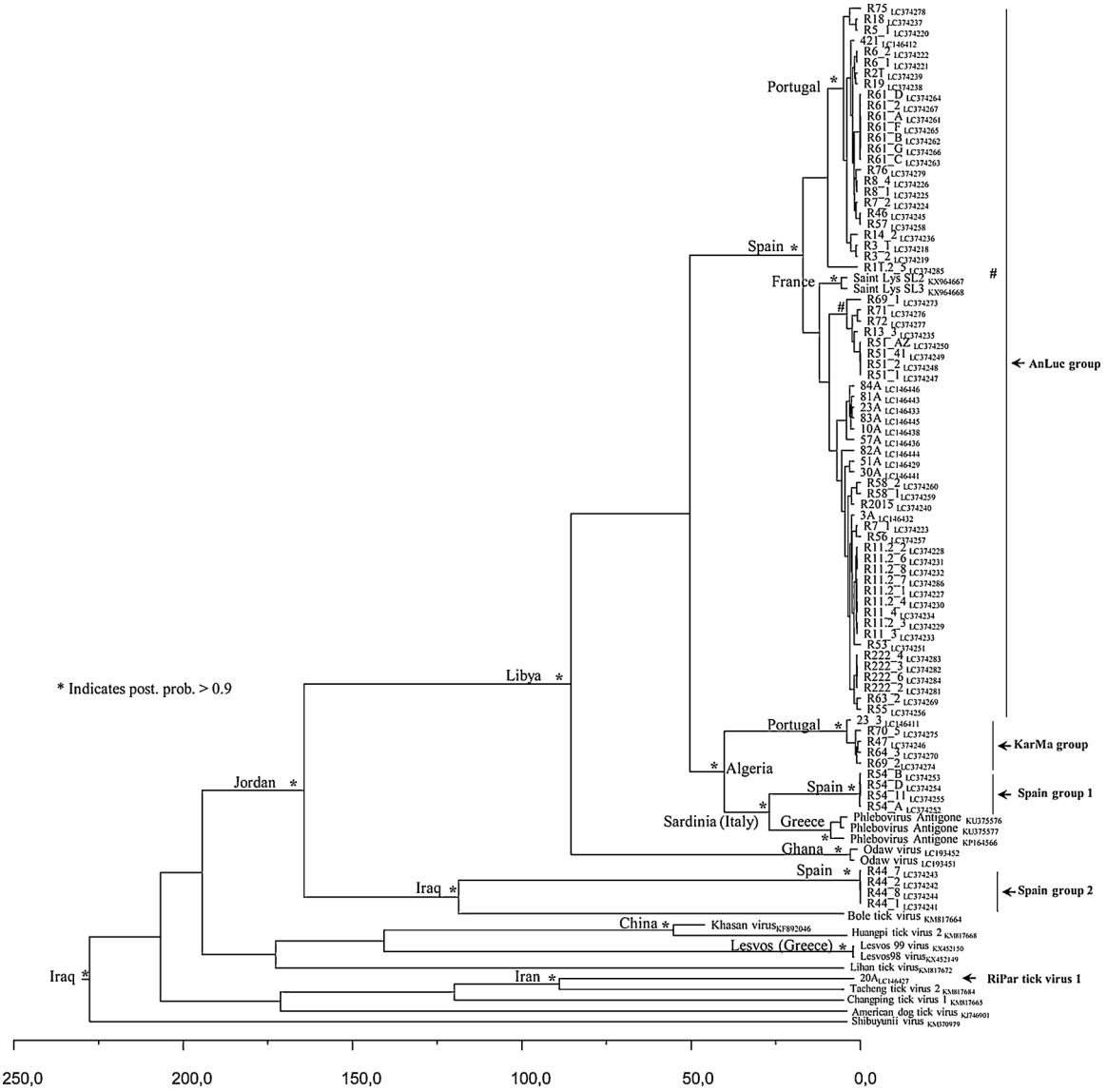

Fig. 2. Time scaled Bayesian phylogenetic tree of Phlebovirus from polymerase-coding sequence (n = 95). At specific branch nodes posterior probabilities ≥0.90 are displayed by *. The most probable geographic location of the ancestors indicated by the different bifurcating nodes is indicated. The reverse time-scale indicates years before the present (data of the most recent sequence). Viral sequences are identified by their name and accession number (underscore).

large monophyletic cluster, along with many others, representing viruses detected over a large geographic area (Al'khovskiĭ et al., 2013; Kobayashi et al., 2017; Li et al., 2015; Keita Matsuno et al., 2015; Papa et al., 2017, 2016; Prinz et al., 2017; Tokarz et al., 2014). Curiously, cloning of a single amplification product obtained from a pool of ticks collected in Portugal revealed that not all of them associate in a single monophyletic group, which suggests that in ticks collected in a small area may harbor viruses which are clearly genetically diverse.

In addition, when primers that have been shown to allow the amplification of S genomic segments of many bunyaviruses, including 29 arthropod-borne human pathogens classified in the genera *Orthobunyavirus*, *Orthonairovirus*, and *Phlebovirus* (Lambert and Lanciotti, 2009), no amplification of any viral sequences was achieved, but these results are not entirely surprising. In fact, it is virtually unfeasible to identify sufficiently conserved segments in multiple sequence alignments of M and S sequences covering the wide range of *Phlebovirus* groups observed in phylogenetic trees (Matsuno et al., 2015), in order to support the design of convenient amplification primers, suggesting that further characterization of these viruses must rely on NGS.

The phylogeographic analysis presented here points to the Middle-East as the most likely place of origin of these putative phleboviruses < 250 years (1630–1893, 95% HPD). Despite the limitations imposed by the small size of the genomic fragment that was analysed, many of the branches in the obtained MCC tree (Fig. 2) were indeed supported by high values (> 0.9) of posterior probability, and indicated that dispersal of these viruses in Europe may also have involved northern Africa.

Although endowed with relatively limited self-dispersal, as ectoparasites ticks are allowed to travel over large distances once attached, for example, to migratory birds. In fact, the viral dispersal routes suggested in this report are clearly compatible with the movement of ticks by birds moving along the Black Sea/Mediterranean and the West Asian-East African flyways (http://wysinfo.com/Migratory_Birds/Migratory_Birds_Without_Boundaries.htm). Alternatively, tick dispersal may also occur once they become attached to those domestic mammals that humans trade, move seasonally (transhumance), or keep as pets. While the geographic dissemination of viruses by ticks attached to domestic animals may be deterred by the implementation of sanitary restrictions/parasite control measures or the attention of the pet

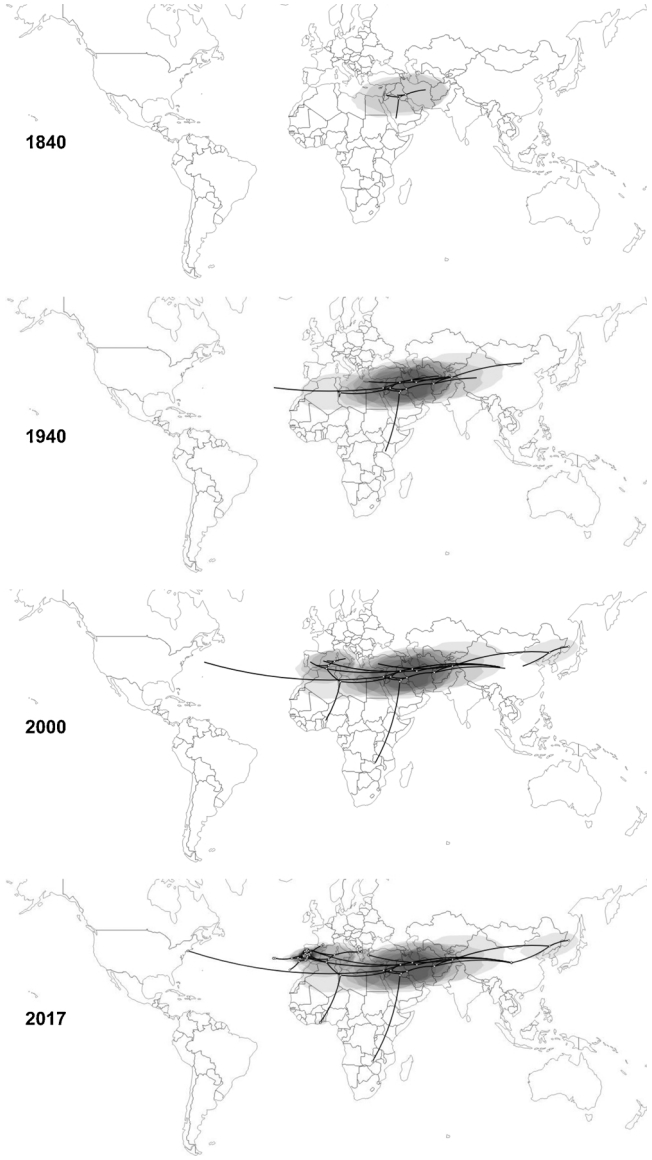

Fig. 3. Different stages of the putative phylogeographic history of the *Phlebovirus* world spread under uncorrelated lognormal relaxed clock priors, and a continuous diffusion model that estimates the ancestral locations of the viruses in continuous space change using changes in coordinates (latitude and longitude). The circular polygon area is proportional to the number of tree lineages maintaining that location.

owners, when ticks are not clearly engorged, they may remain inconspicuous, and are difficult to identify. This piggyback travel capacity of ticks is all the more evident when the immature forms are taken into consideration. Not only are these much more difficult to detect, they also frequently feed on free-roaming small mammals and birds, which may potentially take them over large distances.

Finally, for the time being, many questions remain regarding these putative tick phleboviruses, which will have to await clarification in subsequent studies. Among them are the identification of their putative natural reservoir host and a confirmation of their spatiotemporal dispersal pathways based on the analysis of larger genomic segments and a dataset representing viral sequences sampled over a larger geographic area.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.05.001.

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