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Research paper

New bunya-like viruses: Highlighting their relations

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ABSTRACT

The standard virus classification scheme for arenaviruses and bunyaviruses shifted dramatically when several groups reported the detection and isolation of divergent groups of viruses in a variety of insect collections. Although these viral families can differ in terms of morphology, structure and genetics, recent findings indicate these viruses may have a shared evolutionary origin. To determine the phylogenetic relations among these families, we inferred phylogenetic trees using three methods. The Maximum Likelihood and Bayesian trees were rooted as suggested by the (molecular clock-rooted) BEAST tree. Our results highlight a noteworthy relation among these viral supergroups of different genome organizations. Our study suggests that the best scenario is the existence of at least three monophyletic supergroups, all of them well supported. The recent data indicate that these viruses are evolutionarily and genetically interconnected. While these supergroups appear to be closely related in our phylogenetic analysis, other viruses should be investigated in future research. In sum, our results also provide insights into the classification scheme, thereby providing a new perspective about the fundamental questions of family origins, diversity and genome evolution.

1. Introduction

Most human pathogenic bunyaviruses and arenaviruses cause severe hemorrhagic fevers with a high rate of fatalities. Increasing number of outbreaks and the possibility of cases spreading over international borders has led to increased interest in these viruses and their relations. The ongoing threat of emerging hemorrhagic diseases has made the search for reservoir species with a history of coevolution, for example, with the mammarenaviruses, and hantaviruses, a priority (Charrel and de Lamballerie, 2010; de Oliveira et al., 2014; Zapata and Salvato, 2013). The origin, phylogenetic relationships and evolutionary history of viral genomes is a classic problem that has inspired a long series of questions and hypotheses in evolutionary biology. Recently, sequence analyses of emerging viruses have shown that genes from arenaviruses are potentially homologous to other negative-strand viruses such as bunyaviruses and filoviruses (Carter et al., 2012; Gallaher et al., 2001).

Viruses in the family *Bunyaviridae* (the bunyaviruses) share several molecular characteristics. Based on their differences, the International Committee on Taxonomy of Viruses (ICTV) has classified them into five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (Elliott, 2009). In 1975, this family was proposed to encompass viruses with morphological and structural similarities, but with diverse life cycles. The *Bunyaviridae* genome is comprised of three negative-sense RNA segments (large, medium and small) that employ a variety of coding strategies to generate a limited set of structural and non-structural proteins (Schmaljohn and Nichol, 2007). The large (L) RNA of these viruses codes for the transcriptase and replicase proteins and large RNA-dependent RNA polymerase (RdRp or L protein). Glycoproteins are coded by the medium (M) RNA, which generates a polyprotein that is proteolytically processed. In viruses of some genera, a non-structural protein (NSm) of unknown function is also included. The small (S) RNA codes for the nucleocapsid protein and also for a non-structural protein in viruses of several genera (Elliot and Schmaljohn, 2013; Elliott, 2014). For instance, the L and S segments of the tick-borne Crimean Congo hemorrhagic fever virus (family *Bunyaviridae*, genus *Nairovirus*) encode a polymerase and a nucleocapsid with strong similarity to the Lassa virus (family *Arenaviridae*, genus *Mammarenavirus*) (Carter et al., 2012).

The first discovery of a "virus of experimental lymphocytic choriomeningitis", today well known as the lymphocytic choriomeningitis virus (LCMV), occurred in 1933 (Armstrong and Lillie, 1934). However, only in 1976 was the family *Arenaviridae* established to include the genus *Arenavirus* with LCMV and Tacaribe complexes recognized. Members of the monogeneric family

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Arenaviridae had been reported to infect only rodents, except in one case of bats and ticks (Radoshitzky et al., 2015; Sayler et al., 2014). This scenario shifted dramatically when several groups published the detection and isolation of divergent arenaviruses in captive snakes (Bodewes et al., 2013; Stenglein et al., 2012). The new virus is characterized by ambisense coding and its L and NP genes are homologous to those found in arenaviruses (Zapata and Salvato, 2013). However, the GP sequences are homologous to filovirus envelope glycoproteins (family *Filoviridae*), and the Z gene sequences are homologous to host ubiquitin ligase (Bodewes et al., 2013; Hetzel et al., 2013; Stenglein et al., 2012).

Therefore, arenaviruses are currently classified in two different genera: *Mammarenavirus* and *Reptarenavirus*. Arenaviruses possess single-stranded bi-segmented RNA genomes. Each of the two RNA segments codes for two non-overlapping reading frames of opposite polarities: the viral RNA-dependent RNA polymerase (L protein) and a zinc-binding matrix protein (Z protein) for the large (L) genomic segment. The nucleocapsid protein (NP) and the glycoprotein precursor (GPC) are secondarily cleaved into the envelope proteins G1 and G2 for the small (S) genomic segment (Pinschewer et al., 2003; Qi et al., 2010; Shtanko et al., 2010).

The paradigmatic phylogenetic relation scheme for arenaviruses and bunyaviruses shifted dramatically when several groups independently reported the detection and in some cases the isolation of a divergent group of viruses in a diverse collection of insects, spiders and other arthropods (Frey et al., 2016; Li et al., 2015; Marklewitz et al., 2015). Notably, these new negative-sense RNA viruses were found to be spread across the major lineages of the family *Bunyaviridae*. Although these viral families differ in structure or genetics, these recent findings indicate that these viruses may have a shared evolutionary origin. In this study, we explore key aspects of the evolution of these viruses, particularly their phylogenetic relations, highlighting a perspective in the viral classification scheme, diversity and genome evolution.

2. Materials and methods

2.1. Compiled sequence data

The genomic sequences used in the study were all retrieved from the GenBank® database of NCBI (http://www.ncbi.nlm.nih.gov/nuccore/), including the protein sequences of the full genome of the *Arenaviridae* and *Bunyaviridae* families. Within the *Arenaviridae* and *Bunyaviridae* families, we retrieved representative sequences of each genus due to the very high number of species. We added novel lineages of bunyaviruses that have been discovered in insects, spiders, centipedes and other arthropods that might lead to the establishment of at least eight new genera. We retrieved two genera, also related to bunyaviruses (*Emaravirus* and *Tenuivirus*), that are recognized by the ICTV but have not yet been assigned to a family.

Multiple sequence alignment (MSA) were performed using MAFFT version 7 employing the E-INS-i algorithm and TCOFFEE version 11 applying the PSI-Coffee algorithm (Katoh and Standley, 2013; Notredame et al., 2000). Additionally, we used COBALT, which is a protein multiple sequence alignment tool that finds a collection of pairwise constraints derived from a conserved domain database, the protein motif database, and sequence similarity using RPS-BLAST, BLASTP and PHI-BLAST (Papadopoulos and Agarwala, 2007). The sequence alignment was limited to conserved domains, with ambiguously aligned regions removed using TrimAl (Capella-Gutiérrez et al., 2009). We measured alignment confidence based on a Transitive Consistency Score (TCS) web server. The TCS makes it possible to estimate the local reliability of protein MSAs using the TCS index. The purpose of an alignment reliability index is to discriminate between correctly and incorrectly aligned residues. This evaluation can be used to identify the aligned positions most likely to contain structurally analogous residues, as judged from BAliBASE and PREFAB structure-based reference alignments, and is also most likely to support an accurate phylogenetic reconstruction (Chang et al., 2015; 2014).

2.2. Phylogenetic analyses

We estimated phylogenetic relations of the protein sequences from the three major open reading frames (RdRp, NP and glycoprotein) using (a) ML phylogenetic inference as implemented in PhyML 3 (Guindon and Gascuel, 2003) under the LG + G + I model of sequence evolution, and (b) a Bayesian Markov Chain Monte Carlo (MCMC) method as implemented in MrBayes v3.2.5 (Ronquist et al., 2012). The MCMC settings consisted of two simultaneous independent runs with four chains each that were run for 10 million generations and sampled every 100th generation, yielding 100,000 trees. After eliminating 10% of the samples as burn-in, a consensus tree was built. Statistical support of the clades was measured by a heuristic search with 1000 bootstrap replicates in PhyML (Anisimova and Gascuel, 2006) and the Bayesian posterior probabilities in MrBayes. For the Bayesian analyses, we used a mixed aa model of evolution with γ-shaped distribution of rates across sites. This model allows selection to be integrated across all best-fit models. The best-fit evolutionary model was determined using MEGA version 6.06, using the Bayesian Information Criterion (Tamura et al., 2013).

A rooted timetree (relaxed molecular clock) of amino acid (aa) sequences was inferred using the Bayesian MCMC method available in the BEAST v1.8.4 package. (Drummond and Rambaut, 2007) We modeled the evolutionary rate evolution along branches by an uncorrelated lognormal prior, and the topologies by the Bayesian Skyline tree prior. Two independent runs were undertaken with sampling every 1000 generations. In BEAST, the same evolutionary model was employed as described above. We used Tracer v.1.6 to check for convergence and adequate mixing (i.e., an estimated sample size >200 for all relevant parameters). The TreeAnnotator program was used to generate a Maximum Clade Credibility (MCC) tree after eliminating the first 10% of the sampled trees as burn-in. Because the MCC tree is automatically rooted in the assumption of a molecular clock, it is possible to determine which viral lineages are most likely to be the stem lineage. The stem clade estimated by the BEAST tree was then used as an outgroup to root the phylogenetic trees inferred in the ML and Bayesian phylogenetic analyses.

2.3. Criteria for demarcation of the supergroup

The most universally used sequence-based virus classification tool is phylogenetic analysis. About 70% of the families and floating genera described in the Ninth Report of the International Committee on the Taxonomy of Viruses (ICTV) are supported by phylogenetic trees (King et al., 2012). The chief characteristics of members are presented with phylogenetic analyses of selected genes to support their relations. To help define suitable phylogenetic criteria for relation demarcation, each supergroup was considered to form phylogenetic group when it was clustered into a statistically supported monophyletic clade stem (BEAST/ML/MrBayes, >0.9/>90/>0.9).

3. Results

3.1. Database and multiple sequence alignment

We retrieved a total of 397 sequences from GenBank®: 131 NPs, 119 glycoproteins and 147 L RdRp sequences (Table S1). The aa sequences were aligned using COBALT, MAFFT and TCOFFEE multiple sequence alignment (MSA) methods, and each alignment estimated the positions most likely to contain structurally analogous residues. Starting with the transitive consistency score (TCS) scheme, we selected the MSA to be performed in the MAFFT algorithm.

3.2. Phylogenetic analyses of RdRp sequences

The phylogenetic inference based in the RdRp sequences suggests the existence of at least three monophyletic supergroup, all of them well-supported (Fig. 1). The Maximum Likelihood (ML) and Bayesian trees are rooted in the way suggested by the (molecular clock-rooted) BEAST tree. The first supergroup is bunya-like virus, comprised of one well-supported deeply rooted clade (BEAST/ML/MrBayes = 1/98/1) (Fig. 2). This supergroup is composed of a viral sequence of Wuhan insect virus 3 (GenBank AJG39263), found in the host *Asellus* sp., occupying the basal position in the supergroup. The next bifurcation comprises two well-supported clades, one (1/94/1) of which includes the sequence of Shayang spider virus 2 (AJG39247), found in *Neoscona nautica*, in a sister relation to the clade of the *Jonvirus*, *Feravirus* and *Phasmavirus* genera, all of them well supported as a monophyletic group (1/100/1). The other clade is composed of two phylogroups (1/70/1), the first including the sequence of Whenzhou shrimp virus 2 found in *Penaeus monodon*, occupying the basal position of the phylogroup (0.97/†/0.87). The next bifurcation (*/*/1) within the phylogroup includes the genus *Hantavirus* (1/100/1) and the sequences that form a monophyletic clade (1/100/1), including Shuangao bedbug virus (AJG39248), Shuangao mosquito virus (AJG39252) and Jiangxia mosquito virus 2 (AJG39141), found in *Cimex hemipterus*, *Armigeres subalbatus* and *Culex tritaeniorhynchus*, respectively. The second phylogroup (1/91/1) is composed of the genus *Tospovirus* (1/100/1) as a stem lineage in the phylogroup, with the next bifurcation (0.82/†/0.96) within the phylogroup containing the genus *Emaravirus* (1/100/1), followed by the genera *Orthobunyavirus* and *Herbevirus*, all of them well-supported groups (1/100/1).

The second supergroup is arena-nairo-like viruses, composed of two well-supported deeply rooted clades (1/100/1), one of which includes the two genera *Mammarenavirus* and *Reptarenavirus*, henceforth referred to as arenaviruses (1/100/1). The other clade is from now on referred to as nairoviruses, and is composed of the genus *Nairovirus* (1/100/1). The sequence Jiangxia mosquito virus 1 found in the *Culex tritaeniorhynchus* (GenBank AJG39240), although occupying a stem

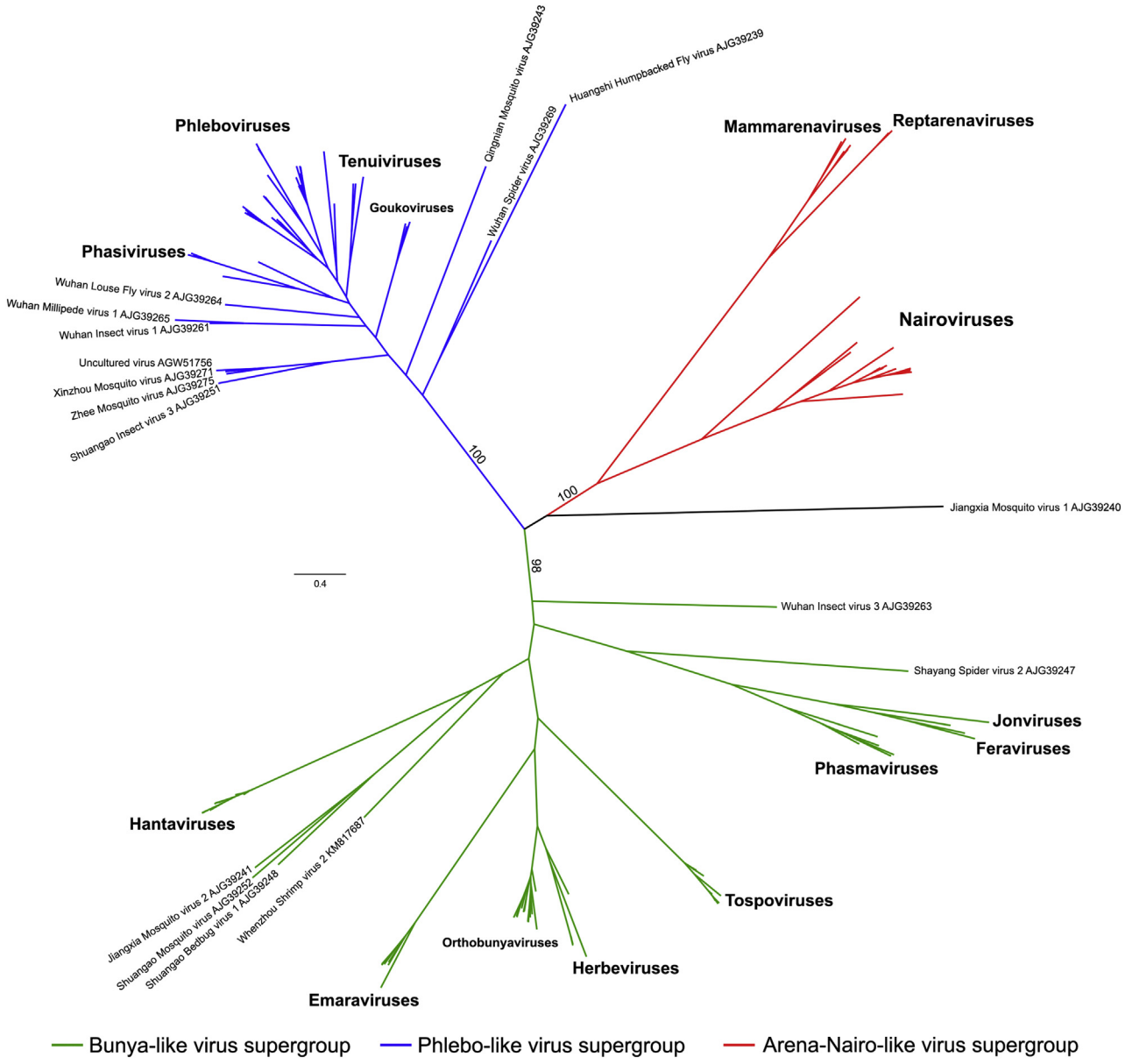

Fig. 1. Unrooted phylogenetic tree of bunya-like viruses based on the RdRp protein sequences, estimated using the ML method. In green, bunya-like virus supergroup; in blue, phlebo-like virus supergroup; and in red, Arena-Nairo-like supergroup. Numbers (>90%) above branches indicate bootstrap values of the supergroups. Scale bar represents number of amino acid substitutions per site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

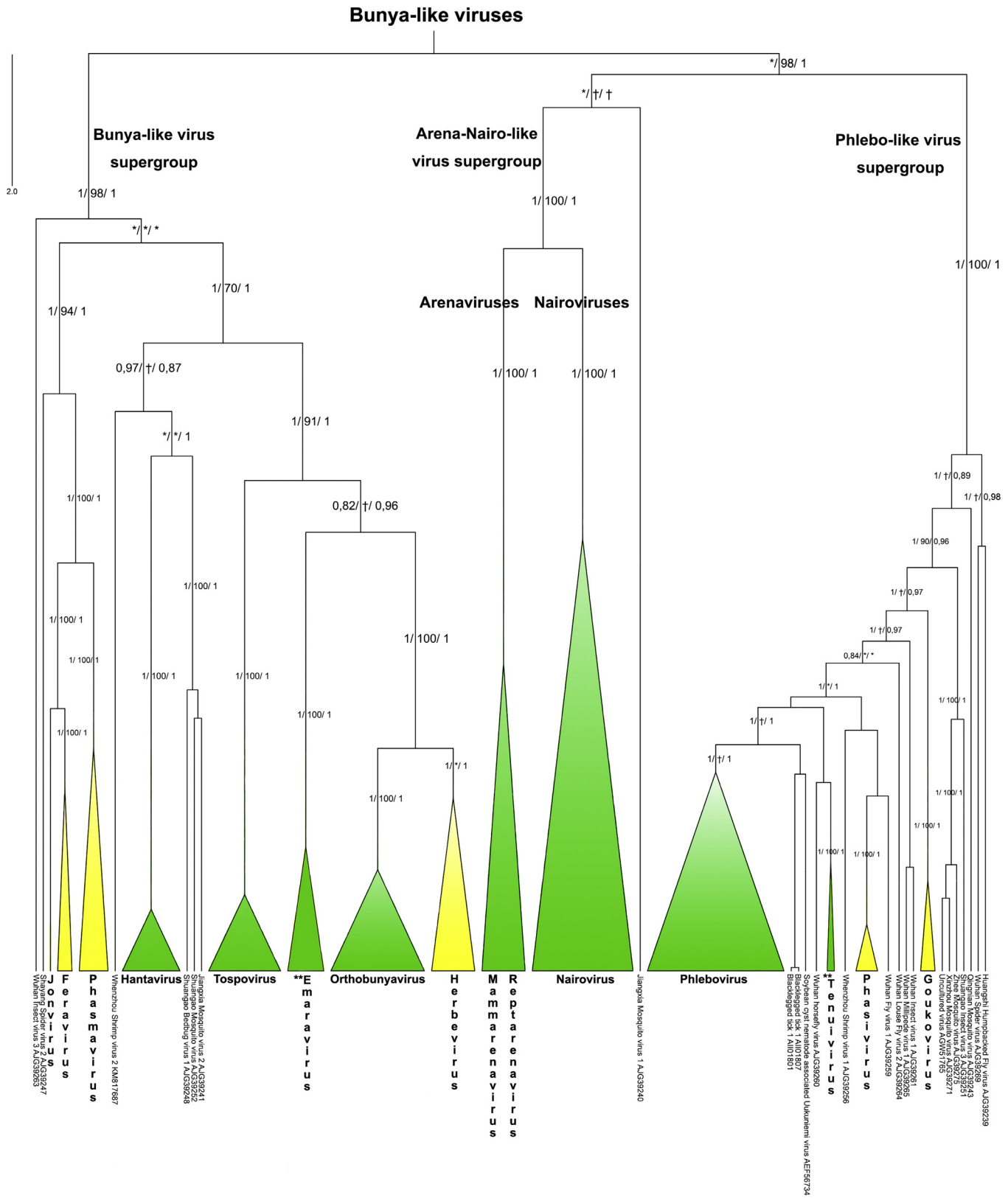

Fig. 2. Phylogenetic trees based on the RdRp protein (604 aa) sequences of arenaviruses, bunyaviruses and novel lineages viral estimated using ML and Bayesian methods. The MCC tree – shown here – was automatically rooted on the assumption of a molecular clock. The basal lineage estimated by the MCC tree was then used as an outgroup in the Bayesian and ML analyses. Numbers (>0.7/>70%/>0.7) above branches indicate posterior node probabilities or bootstrap values (BEAST/ML/Mrbayes). *Indicate values below 0.7/70. †Exhibited difference between BEAST, ML trees and MrBayes tree-building method topology. Green indicates genera recognized by the ICTV and yellow indicates genera that have been proposed by the ICTV. **The genus is recognized by the ICTV, but has not yet been assigned to a family. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lineage to the arenaviruses, has uncertain basal position in the phylogenetic tree (*/†/†). The ML and MrBayes exhibit differences compared with the BEAST, and the Jiangxia mosquito virus 1 sequence occupies a stem lineage to the third family, referred to here as phlebo-like supergroup.

The phlebo-like virus supergroup is composed of one well-supported deeply rooted clade (1/100/1). Although this supergroup is well-supported as being a monophyletic clade in three phylogenetic methods, some of its relations in the next bifurcations are uncertain. The ML exhibited differences when compared with BEAST and MrBayes. The Huangshi humpbacked fly virus (AJG39239) found in unidentified Phoridae and the Wuhan spider virus (AJG39269) found in *Neoscona nautica* form a clade (1/†/0.98) that occupies a stem lineage to the phlebo-like supergroup, followed by the Qingnian mosquito virus (AJG39243) sequence found in *Culex quinquefasciatus*, comprising the stem sister clade (1/†/0.89). The next bifurcation (1/90/0.96) is formed by a monophyletic clade (1/100/1) comprised of Shuangao insect virus (AJG39251), Zhee mosquito virus (AJG39275), Xinzhou mosquito virus (AJG39271) and an uncultured virus (AGW51765) found in unidentified Chrysopidae, *Anopheles sinensis* (Zhee and Xinzhou) and *Culicine* sp., respectively. The next bifurcation (1/†/0.97) is comprised of the monophyletic genus *Goukovirus* (1/100/1), being an addition to the next bifurcation, containing a clade formed by Wuhan insect virus (AJG39261) and Wuhan millipede virus 1 (AJG39265), found in *Asellus* sp. and unidentified Polydesmidae, respectively. The Wuhan Louse fly virus 2 (AJG39264), found in unidentified Hippoboscoidea, comprised the stem sequence of the next bifurcation, although having an uncertain phylogenetic position (0.84/*/*), being close to a clade composed of three indoor clades. The first clade is formed by Whenzhou shrimp virus 1 (AJG39256) and the phylogroup composed of the genus *Phasivirus* (1/100/1) and Wuhan fly virus 1 (AJG39259), found in *Atherigona orientalis*. The second clade consists of the genus *Tenuivirus* (1/100/1) and Whenzhou shirmp virus 1 (AJG39256), found in *Penaeus monodon*. The third clade (1/†/1) is comprised of the genus *Phlebovirus* (1/†/1) and blacklegged tick 1 and 2 viruses (AII01807, AII01801), found in *Ixodes scapularis* plus the Soybean cyst nematode associated with Uukuniemi virus (AEF56734), found in *Heterodera glycines*.

3.3. Phylogenetic analyses of NP sequences

The phylogenetic relations based on the NP sequences revealed minor differences compared with the RdRp trees, with relatively strong statistical support in some branches. Because of the high genetic diversity, we conducted the NP analyses by related groups within of the bunya-like virus supergroup. In the NP sequence tree of the first group, we evaluated *Phasmavirus*, *Feravirus* and *Jonvirus* genera (Fig. 3). The same topology of the L protein tree was found, with the *Feravirus* and *Jonvirus* genera branched monophyletically (1/100/1) subsequent to the clade of the genus *Phasmavirus* (0.70/99/1). The second group, formed by the genus *Hantavirus* and a new viral sequences found in invertebrates, was not included in the NP analyses due to the lack of NP sequences of these new invertebrate viruses. The third group, composed of the genera *Tospovirus*, *Emaravirus*, *Orthobunyavirus* and *Herbevirus* (Fig. 4), recovered as a monophyletic group (1/100/1) from the genus *Emaravirus* (plant-pathogenic viruses), was now found occupying a stem position in the group, different from what was retrieved in the polymerase tree. In the sister clade (0.74/100/1), the first bifurcation was comprised of the genus *Tospovirus* (1/100/1), followed by Shuangao insect virus 1 (AJG39311) (*/†/†) and the next bifurcation, composed of the genus *Herbevirus* (*/†/†), although having uncertain phylogenetic position. The Akhtuba virus (AIL53815) and Khurdun virus (AHL27171) formed a monophyletic clade (1/100/1), which was an uncertain sister clade (*/†/†) of the genus *Orthobunyavirus* (1/70/0.98). It is important to note that the ML and Bayesian tree topologies were different (†) in poorly supported branches. The main difference between the NP and L protein phylogenetic trees were the Shuangao insect virus 1, Akhtuba virus and Khurdun virus, which do not form a monophyletic clade within the genus *Herbevirus*.

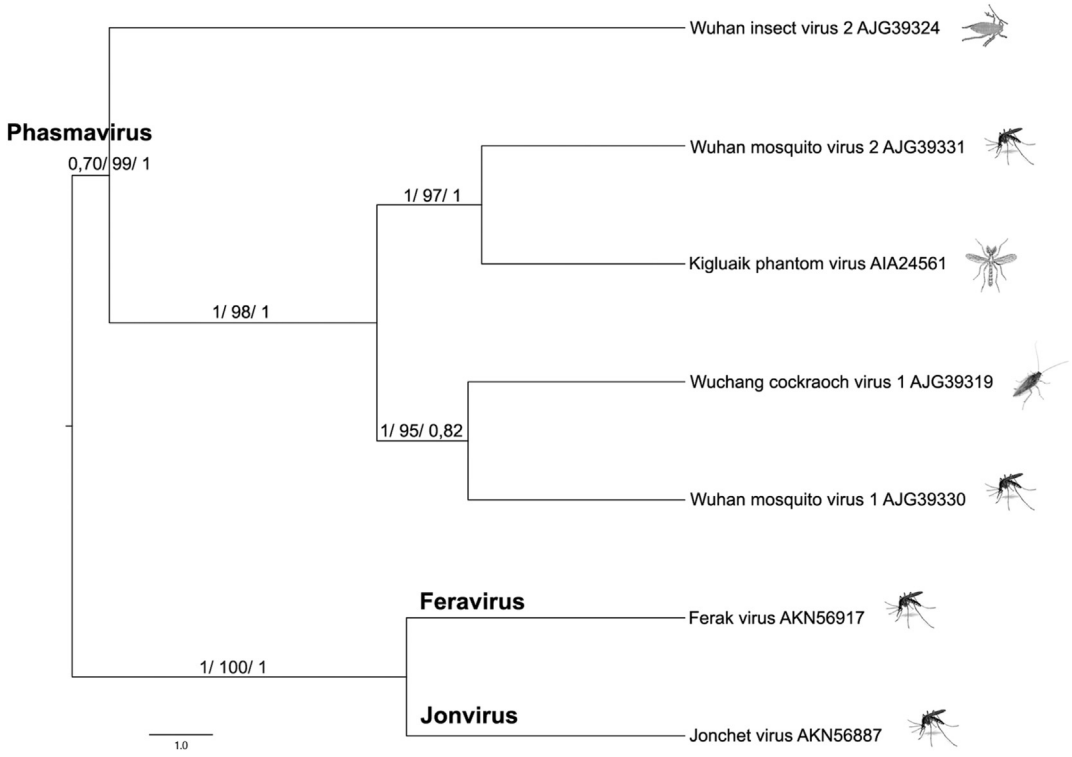

Fig. 3. Phylogenetic trees based on the NP protein (263 aa) sequences of feraviruses, jonviruses and phasmaviruses, estimated using ML and Bayesian methods. The MCC tree – shown here – was automatically rooted on the assumption of a molecular clock. The basal lineage estimated by the MCC tree was then used as an outgroup in the Bayesian and ML analyses. Numbers (>0.7/>70%/>0.7) above branches indicate posterior node probabilities or bootstrap values (BEAST/ML/Mrbayes). *Indicates values below 0.7/70. †Exhibited difference between BEAST, ML trees and MrBayes tree-building method topology. Illustration of some reservoirs/hosts.

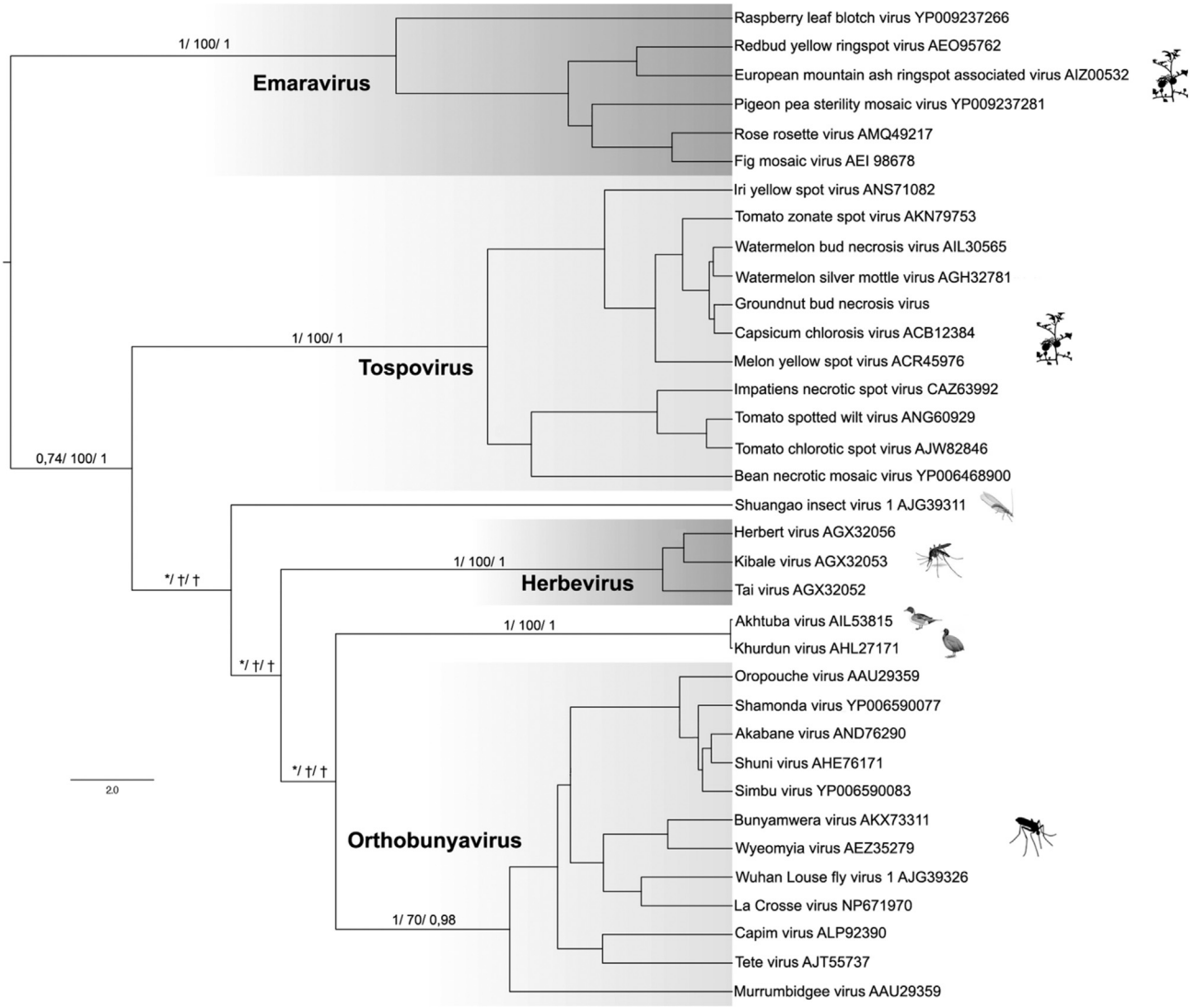

Fig. 4. Phylogenetic trees based on the NP protein (166 aa) sequences of emaraviruses, herbeviruses, orthobunyavirus and tospoviruses, estimated using ML and Bayesian methods. The MCC tree – shown here – was automatically rooted on the assumption of a molecular clock. The basal lineage estimated by the MCC tree was then used as an outgroup in the Bayesian and ML analyses. Numbers (>0.7/>70%/>0.7) above branches indicate posterior node probabilities or bootstrap values (BEAST/ML/Mrbayes). *Indicates values below 0.7/70. †Exhibited difference between BEAST, ML trees and MrBayes tree-building method topology. Illustration of some reservoirs/hosts.

The phylogenetic inference based on the NP sequences of the arenanairo-like virus supergroup produced highly similar topologies in relation to the L sequence tree (Fig. 5). The nairoviruses were recovered as a monophyletic clade (1/100/1). This group was composed of Shayang spider virus 1 (AJG39310), found in the host *Neoscona nautica* occupying the basal position in the nairoviruses. The next bifurcation is formed by a monophyletic clade (1/100/1), which is comprised of Wuhan millipede virus 2 (AJG39329), Sanxia water strider virus 1 (AJG39309) and Xinzhou spider virus (AJG39333), found in unidentified Polydesmidae, unidentified Gerridae and *Neoscona nautica*, respectively. The next bifurcation (1/95/1) is comprised of a monophyletic clade of the viruses found in ticks. Within the arenaviruses (1/100/1), as expected the genus *Reptarenavirus* (1/100/1) formed one monophyletic group that is deeply rooted to the genus *Mammarenavirus* (1/86/0.99). Mammarenaviruses were divided into two distinct groups. Old World and New World mammarenaviruses fall into two separate groups, corresponding to the Lassa-Lymphocytic choriomeningitis virus (1/93/1) and Tacaribe serocomplexes (1/86/0.99), respectively. The NW mammarenaviruses were divided into three lineages, also known as clades A, B and C, which were well-supported as monophyletic.

The final alignment length of the family phlebo-like virus supergroup was 69 amino acids for NP datasets. This reduced size was uninformative, so in our phylogenetic analysis the effective sample size (ESS) of some parameters was under 200, indicating parameters did not converge (data not shown). Trees based on glycoprotein sequences were affected in our phylogenetic inference. All of the species included in this study clearly did not constitute a distinct monophyletic genus. The tree topologies that we estimated using glycoproteins were not consistent, indicating that the distinctly varied sizes of proteins adversely affected our phylogenetic analyses.

4. Discussion

The classification of bunyaviruses does not obey any strictly defined criteria, although they are unified by common characteristics, such as having a single strand with negative sense, a segmented RNA genome, an enveloped, spherical virion of about 80–120 nm in diameter, a virion composed of four structural proteins, a cytoplasmic site of replication and intracellular budding at the Goldi complex. Mature virions contain two glycoproteins, named Gn and Gc, which are embedded in the viral envelope, a nucleocapsid (N) protein that forms a complex with the

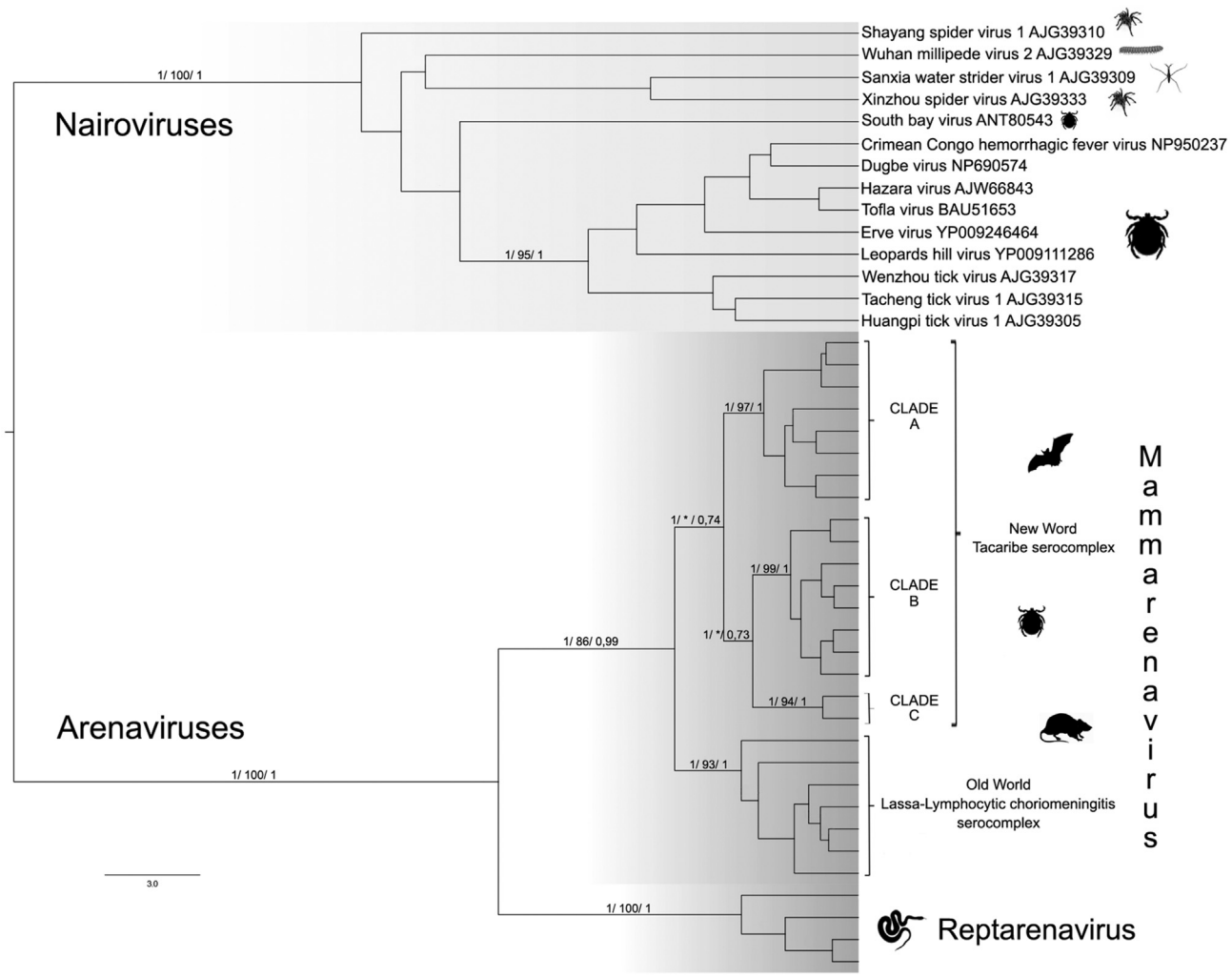

Fig. 5. Phylogenetic trees based on the NP protein (299 aa) sequences of arenaviruses and nairoviruses estimated using ML and Bayesian methods. The MCC tree – shown here – was automatically rooted on the assumption of a molecular clock. The basal lineage estimated by the MCC tree was then used as an outgroup in the Bayesian and ML analyses. Numbers (>0.7/>70%/>0.7) above branches indicate posterior node probabilities or bootstrap values (BEAST/ML/Mrbayes). *Indicates values below 0.7/70. Illustration of some reservoirs/hosts.

RNA genome segments, called the ribonucleoprotein complex (RNP), and an RNA-dependent RNA polymerase named L protein, which contains an N-terminal endonuclease domain (Elliot and Schmaljohn, 2013; Elliott, 2014; Plyusnin and Elliot, 2011). However, bunyaviruses show major differences in biological behavior and replication strategy. Moreover, the distinguishing features among members of the five genera are differences in the size of genome segments and proteins (for example, L protein of genus *Nairovirus* with about 12.2 Kb–450 kDa and genus Hantavirus 6.5 Kb–250 kDa), different consensus terminal sequences and different coding strategies for the additional nonstructural proteins NSs and NSm. Additionally, host and vector associations vary among the genera [1,3]. Therefore, within the family of *Bunyaviridae*, tospoviruses are the only ones that infect plants and are transmitted by thrips (Rotenberg et al., 2015). Viruses of the other genera infect vertebrates and are transmitted by blood-feeding arthropods, with the exception of hantaviruses. Nairoviruses are transmitted by ticks (Messina et al., 2015), orthobunyaviruses mainly by mosquitoes, and phleboviruses by sandflies, mosquitoes and ticks (Horne and Vanlandingham, 2014). Human infections by hantaviruses result from exposure to aerosolized rodent excreta containing pathogenic viruses, and they are associated with multiple species of rodents, shrews, moles and bats (de Oliveira et al., 2014).

Biologically, these viruses are fairly diverse and have been reported in a wide range of host species. In this study, we compared the genomes of arenaviruses, bunyaviruses and divergent group of novel viruses to assess their relations. Despite the complex evolutionary histories of these viruses, the phylogenetic signals are coherent enough to obtain a consensus phylogeny, which unequivocally points to the common ancestry of these viruses. In summary, recent studies have led to the accumulation of compelling evidence that points to a common ancestry of the arena-nairo-like virus supergroup, bunya-like supergroup and phlebo-like supergroup. Within the bunya-like viruses, each of the supergroups comes across as a clade assembled into four major branches, one of which encompasses the bunya-like supergroup, the second one the arena-nairo-like supergroup formed by the arenaviruses and nairoviruses, the third branch consisting of Jiangxia mosquito virus 1, with uncertain basal position in the phylogenetic tree, and the fourth consisting of the phlebo-like supergroup. Several groups have independently published phylogenetic analyses with a large part of these viruses, providing similar topologies (Junglen, 2016; Li et al., 2015; Marklewitz et al., 2015). However, in none of these studies did the authors use all the new genera and/or new viral sequences, a employed in this study.

The new bunya-like viruses display a wide variety of genome organizations, including two, three or four segments, besides several differences in biological behavior and replication strategy (Elliot and Schmaljohn, 2013; Guu et al., 2012; Mielke-Ehret and Mühlbach, 2012; Plyusnin and Elliot, 2011). Recently, Li and collaborators proposed a novel viral family, the *Chuviridae*. Although monophyletic, the chuviruses display a wide variety of genome organizations including

unsegmented, bi-segmented and circular forms (Li et al., 2015). The phylogenetic tree of evolutionary relations between many groups of negative-sense RNA viruses (including arena-, bunya- and filovirus) suggests a division between the segmented and unsegmented viruses. The identification of this diverse virus family provides a new perspective on the virus classification scheme, which opens up an important line of future research (Dudas and Obbard, 2015). The *Bunyaviridae* family is undergoing a thorough taxonomic revision by the ICTV, and in the next release it will become an order (*Bunyavirales*) with eight families: *Feraviridae*, *Fimoviridae*, *Hantaviridae*, *Jonviridae*, *Nairoviridae*, *Phasmaviridae*, *Phenuiviridae* and *Tospoviridae* (International Committee on Taxomomy of Viruses, 2016). Although bunya-like viruses are well represented in the proposal, arenaviruses are not included yet in this new taxonomic order. According to our results and other studies, *Arenaviridae* should be considered in future taxonomic changes as a possible family or genus related to Nairo-like bunyaviruses. Furthermore, with the inclusion of arenaviruses, three large branches are created, which can change all taxonomic proposals.

Even more convincing evidence for the evolutionary link between arenaviruses and nairoviruses has come from phylogenetic analysis of the NP and L protein. Despite the pronounced differences in genome organization and complexity, members of the two families have an unexpected evolutionary link. It has become apparent that at the genome level nairoviruses are no more similar to the other members of the bunya-like viruses than to the arenaviruses. Recently, a study analyzed a novel bunyavirus group from mosquitoes (Marklewitz et al., 2015). These authors evaluated the relations between novel bunyaviruses with other bunyaviruses and arenaviruses. The genus *Nairovirus* was placed in a basal relation compared with all other bunyaviruses and was considered to be closely related to arenaviruses. As proposed earlier, our trees rooted in a way that suggested that the (molecular clock-rooted) arenaviruses are closely related to the nairoviruses (Carter et al., 2012; Marklewitz et al., 2015; Vieth et al., 2004). However, these groups do not take a basal position with respect to the remaining bunyaviruses. In general, the phylogenetic grouping of nairoviruses that are very well supported with sister clades to the arenaviruses support the view that nairoviruses and arenaviruses are a supergroup. Nairoviruses include many species, all of which are either maintained by arthropods or transmitted by ticks through bats, birds, eulipotyphla or rodents (Kuhn et al., 2016; Plyusnin and Elliot, 2011). Arenaviruses, on the other hand, have been associated mainly with rodents and more recently to boid snakes (Zapata and Salvato, 2013). However, the Tacaribe virus is a mammarenavirus for which a rodent host has never been specified. This virus was first isolated from bats and mosquitoes, and since that one-time isolation, it has not been isolated from any vertebrate or invertebrate hosts (Downs et al., 1963). Recently, the Tacaribe virus was isolated from a pool of *Amblyomma americanum* (lone star ticks) and its complete genome was sequenced (Sayler et al., 2014). This fact leads us to believe that the answer for other mammarenaviruses without a defined reservoir, such as the Sabiá virus, Lujo virus and Chapare virus, can be found in arthropods (Briese et al., 2009; Delgado et al., 2008; Gonzalez et al., 1996). This notable feature highlights an important evolutionary link between nairoviruses and arenaviruses, the invertebrates hosts.

Importantly, in the bunya-like virus supergroup and phlebo-like virus supergroup, our phylogenetic analysis revealed no particular trend for the *Emaravirus, Tospovirus* and *Tenuivirus* genera (plant pathogenic viruses) to form a stem lineage. However, it has been proposed that RNA viruses of plants were acquired through three processes that contributed to the evolution of related viruses in plants and animals: i) evolution from a common ancestral virus predating the divergence of plants and animals; ii) horizontal transfer of viruses, for example, through insect vectors; and iii) parallel origin from related genetic elements (Dolja and Koonin, 2011). This scenario that RNA viruses of plants were acquired from animals via horizontal virus transfer is compatible with the markedly higher diversity and prevalence of animal RNA viruses compared to the relative scarcity of these viruses in plants (Guu et al., 2012).

A different method uses the fossil record of the hosts groups to calibrate viruses' evolutionary rates (Bennett et al., 2014). Hantaviruses exhibit an astonishing degree of phylogenetic correspondence with their hosts. These important findings are an indication that the full time scale of hantavirus evolution has been appreciated only now (Guo et al., 2013; Guterres et al., 2015; Holmes and Zhang, 2015). Recently, researchers have proposed that hantaviruses are very ancient viruses that already existed at the estimated diversification point of major placental clades, a diversification that occurred approximately 90–-100 million years ago (Plyusnin and Sironen, 2014). However, it is logical to assume that the evolutionary history of hantaviruses is even deeper in the past, and should include transmission of an ancestral hantavirus harbored by an insect to a mammalian host. Our studies (molecular clock-rooted) also provide important insights into the hantaviruses, which seem to be closely related to new viruses discovered in invertebrates, including one found in shrimp. One notable feature is that the Wuhan insect virus 3 found in *Asellus* sp. (isopod crustaceans) is a stem lineage of the bunya-like virus supergroup. Accordingly, the current study provides a tempting evidence to speculate could bunya-like viruses first appeared in the sea? Indeed, the close phylogenetic relationships among some viruses found in marine invertebrates within the tree supports the occurrence posterior evolutionary events (spillover, host switching) between terrestrial hosts and marine invertebrates.

Determining the date of bunya-like viruses' origin and the rates of their diversification into major lineages correlated with host lineages is challenging given the absence of an independent method of calibration. Some studies have identified endogenous viral elements (EVEs) over 10 non-retroviral families in the eukaryote genome. When the attachment occurs, the integrated endogenous virus genomes evolve at the same host mutation rates, and their sequence is likely to be stably preserved. Therefore, the genomic fossil record may represent an alternative for studying viral evolutionary history on timescales spanning millions of years (Feschotte and Gilbert, 2012; Johnson, 2010; Katzourakis, 2013). In 2009, Geuking and collaborators performed an illegitimate recombination between an exogenous non-retroviral RNA virus, LCMV (genus *Mammarenavirus*) and an endogenous IAP retrotransposon, which led to the reverse transcription of exogenous viral RNA. The resulting complementary DNA was integrated into the host's genome with an IAP element (Geuking et al., 2009). The RNA virus has been found in the genomes of mammal and insect vectors associated with a total of seven viral families, including segmented (*Orthomyxoviridae*, *Bunyaviridae*) and non-segmented (*Bornaviridae*, *Filoviridae* and *Rhabdoviridae*) negative-sense RNA viruses. EVEs have already been identified in nairoviruses and *phleboviruses* (Katzourakis and Gifford, 2010). Interestingly, novel sequences have been characterized from arthropods, and they are most closely related to hantaviruses, reinforcing that the evolutionary history of hantaviruses is even deeper in the past. These data are striking and indicate that the origin of hantavirus may be arthropods (Li et al., 2015; Marklewitz et al., 2015).

An understanding of the timescale of evolution is critical for comparative virology but remains elusive for many RNA viruses. For example, molecular estimates of the age of the common ancestor of known filoviruses fall into two time ranges. One range is coincident with the rise of agriculture, from 7100 to 10,400 years ago (Carroll et al., 2013; Li and Chen, 2014; Suzuki and Gojobori, 1997). The other range is from the Middle Pleistocene 155,000 years ago (Negredo et al., 2011) or from the orthologous filovirus EVEs, which have been described in mouse and rat genomes. These data yield a minimum age of 30 million years for the family *Filoviridae* (Belyi et al., 2010; Taylor et al., 2010). A leading example is foamy viruses, complex retroviruses that infect a variety of placental mammals (Wu et al., 2012). Initially, a comparison of the phylogenies of simian foamy viruses and OW primates suggested that they co-speciated with each other for

>30 million years (Switzer et al., 2005). However, the recent discovery of endogenous foamy virus-like elements in the genome of the coelacanth (*Latimeria chalumnae*) suggests that foamy viruses and their vertebrate hosts likely codiverged >407 million years ago (Han and Worobey, 2012).

5. Conclusion

An important question in the arenaviruses, bunyaviruses and novel related lineages —and the source of considerable debate—is the evolutionary history of the events discussed here. Our results highlight the remarkable relation among these viral supergroups of different genome organizations. Our study suggests that the best scenario is the existence of at least three monophyletic supergroups, all of them well supported. The first bunya-like virus supergroup is composed of emaraviruses, feraviruses, jonviruses, hantaviruses, herbeviruses, orthobunyaviruses, phasmaviruses and tospoviruses. The arena-nairo-like supergroup exhibited a statistically supported monophyletic cluster, reinforcing the inclusion of arenaviruses in the bunyavirus group. This notable phylogenetic relationship can be the answer for mammarenaviruses without a defined mammalian reservoir, which can be found in arthropods. Lastly, a phlebo-like virus supergroup arises, formed by goukoviruses, phasmaviruses, phleboviruses, tenuiviruses and the divergent group of viruses not assigned to a genus. An important piece of evidence revealed by our phylogenetic analyses was the absence of a particular stem lineage trend for the *Emaravirus*, *Tospovirus* and *Tenuivirus* genera (plant pathogenic viruses). Accordingly, in order to learn the evolutionary relationship between segmented viruses of the three supergroups, and because of the wide diversity (genome organizations, replication strategy, hosts and among others), we named them “bunya-like viruses”. The recent data reinforce that these viruses are evolutionarily and genetically interconnected. While these supergroups appear to be closely related in our phylogenetic analysis (molecular clock-rooted), other viruses should be investigated in future research. Our results also provide insights into the classification scheme, thereby providing a new perspective about the fundamental questions of family origins, diversity and genome evolution.

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Competing interests

No competing financial interests exist. The authors have no conflicts of interest or disclosures to make concerning this work.

Author contributions

Conceived and designed the experiments: AG. Performed the experiments: AG CGS. Analyzed the data and interpretation: AG RCO JF ERS CGS. Wrote the paper: AG CGS. Revised the manuscript: AG RCO JF ERS CGS.

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