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Arenavirus genomics: novel insights into viral diversity, origin, and evolution

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Next-generation sequencing technologies have revolutionized our knowledge of virus diversity and evolution. In the case of arenaviruses, which are the focus of this review, metagenomic/metatranscriptomic approaches identified reptile-infecting and fish-infecting viruses, also showing that bi-segmented genomes are not a universal feature of the *Arenaviridae* family. Novel mammarenaviruses were described, allowing inference of their geographic origin and evolutionary dynamics. Extensive sequencing of Lassa virus (LASV) genomes revealed the zoonotic nature of most human infections and a Nigerian origin of LASV, which subsequently spread westward. Future efforts will likely identify many more arenaviruses and hopefully provide insight into the ultimate origin of the family, the pathogenic potential of its members, as well as the determinants of their geographic distribution.

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Introduction

In recent years, the advent of metagenomic and metatranscriptomic approaches resulted in the identification of an outstanding number of novel viruses (e.g., [1••,2••]). Also, next-generation technologies have allowed the rapid sequencing of multiple genomes of known viruses. For instance, the sequencing of Ebola virus (EBOV) and Lassa virus (LASV) from infected patients/animals has provided invaluable information about the genetic diversity and evolutionary dynamics of these human pathogens [3••,4]. In this review, we focus on *Arenaviridae*, a family of negative-sense RNA viruses which includes LASV. We discuss how next-generation approaches and genome sequencing in general have expanded our knowledge about arenavirus diversity, evolution, origin, and host association.

An expanding family with remarkable genome plasticity

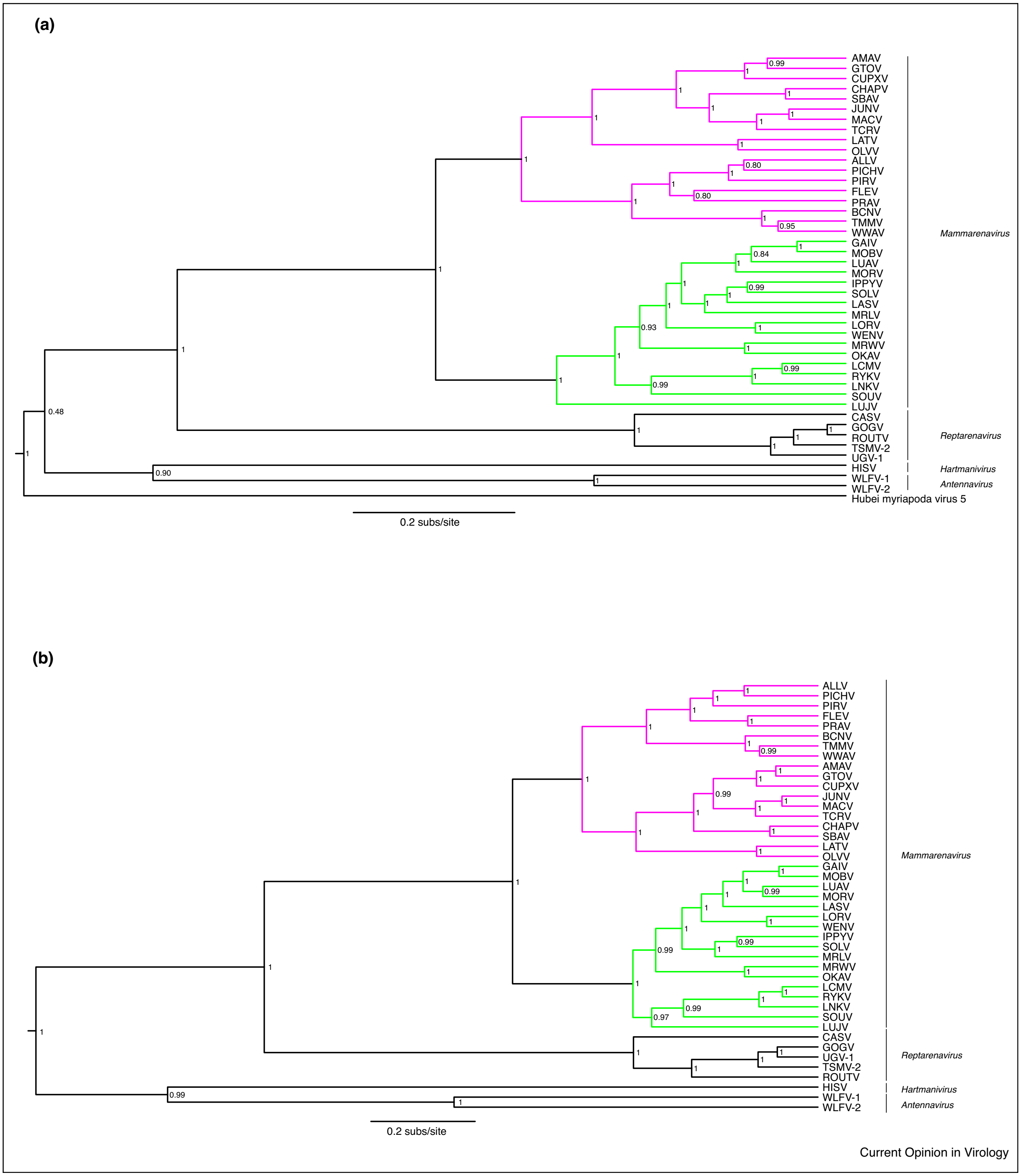
Since 1976 and until 2012 the *Arenaviridae* family comprised a single genus of mammal-infecting viruses now known as *Mammarenavirus*. Most mammarenaviruses have natural reservoirs in rodents and were historically divided into two monophyletic groups: the Old World (OW) and the New World (NW) complexes (Figure 1).

In 2009, several snakes hosted in an aquarium in San Francisco were diagnosed with a fatal condition known as inclusion body disease (IBD). The availability of unbiased methods for pathogen identification, the first representative of which was the Virochip DNA microarray technology [5], spurred the search for the infectious agent (see https://www.ucsf.edu/news/2012/08/12545/mysterious-snake-disease-decoded for a narration of the events). Eventually, in 2012–2013, metagenomic analyses led to the sequencing and subsequent isolation of novel arenaviruses from captive alethinophidian snakes suffering from IBD [6–8]. These works resulted in the establishment of two novel genera (*Reptarenavirus* and *Hartmanivirus*) in the *Arenaviridae* family [9•] (Figure 1).

Mammarenaviruses, reptarenaviruses, and hartmaniviruses have characteristic bi-segmented genomes with ambisense organization. The small segment (S) encodes the glycoprotein precursor (GPC) and the nucleocapsid protein (NP); the large segment (L) codes for the RNA-dependent RNA polymerase (L) and, in the case of mammarenaviruses and reptarenaviruses, for a zinc-binding protein (Z) (Figure 2a). During the life cycle of mammarenaviruses, the matrix Z-protein participates to different processes, including the formation of infectious viral particles and viral budding [10–15]. Current evidence however suggests that hartmaniviruses lack a Z protein [16•], indicating that arenaviruses may have evolved diverse mechanisms of virion assembly and cell egress.

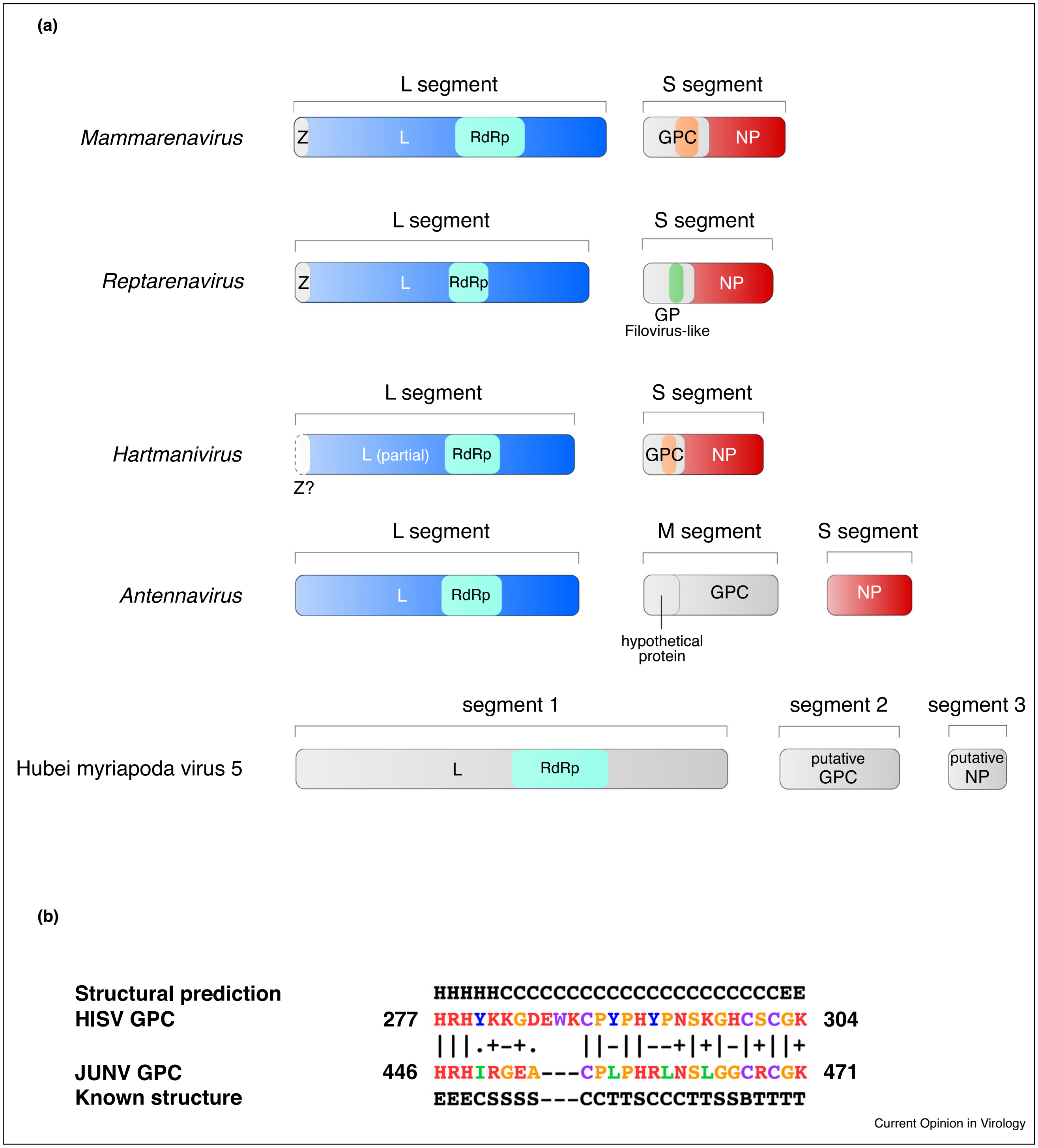
Also, very recent work showed that a bi-segmented genome is not a universal feature of arenaviruses. A large-scale metatranscriptomic study of RNA viruses in cold-blooded vertebrates identified a plethora of novel viruses, including two frogfish (*Antennarius striatus*) arenaviruses with tri-segmented genomes (Figures 1 and 2a) [2••]. Based on sequence similarity and phylogenetic

Figure 1



Phylogenetic relationships for the *Arenaviridae* family.
Maximum clade credibility tree of **(a)** the RdRp domain and **(b)** the NP protein. Only ICTV (International Committee on Taxonomy of Viruses)-approved viral species are included, plus the Hubei myriapoda virus 5 for the RdRp tree. Trees were obtained with BEAST (version 2.4.4) [64] and numbers indicate the node posterior probability. A list of species and virus abbreviations is reported in Supplementary Table 1.

Figure 2



Arenavirus genome organization and sequence similarity.
(a) Arenavirus genome organization is schematically shown together with information on sequence homology. Homologous regions are indicated in the same color; however, color codes and color intensity are arbitrary and not indicative of the level of sequence similarity. Gray indicates regions that display no homology to known proteins outside the genus (as assessed by BLASTp, HHpred and Phyre^2) [65–67]. **(b)** Sequence/structural homology between HISV and Junin virus glycoproteins as detected by Phyre^2 (very similar results were obtained with HHpred, not shown). Phyre^2 predicted JUNV GPC (PDB ID: 2L0Z) as the best match with a confidence of 93.3%. The amino acid alignment is shown with residues colored on the basis of physico-chemical properties. Known and predicted secondary structures are also indicated (H: helix, E: extended strand in β-sheet conformation, C: coil, T: hydrogen bonded turn, B: residue in isolated β-bridge, S: bend).

relationships, the frogfish viruses were proposed to form a new genus (*Antennavirus*) in the *Arenaviridae* family (https://talk.ictvonline.org/files/proposals/animal_dsrna_and_ssrna-_viruses/m/animal_rna_minus_ec_approved). Interestingly, large scale analyses in invertebrates also identified arenavirus-like sequences: the so called Hubei myriapoda virus 5 [1••] shows sequence similarity to arenavirus L proteins and has a tri-segmented genome (Figures 1a and 2 a). Whereas it is presently impossible to determine whether the arenavirus ancestor had a tri-segmented or bi-segmented genome, differences in genome organization indicate plasticity in terms of segment number and, to a lesser extent, gene content (Figure 2a). Metagenomic analyses of reptarenavirus-infected snakes also uncovered an unexpected aspect of arenavirus genomic diversity: the very frequent occurrence of mixed infections and the different representation of S and L segment genotypes [16•,17,18•]. Typically, infected reptiles harbor multiple S and/or L segment genotypes and, in individual snakes, L segment genotypes tend to outnumber S segment genotypes.

Overall, L segments are the most genetically diverse and distinct S and L genotypes are detected at different frequencies in snake populations. Recent data [19] indicated that this situation is likely to be caused by competition among segment genotypes, which may either occupy different fitness landscapes or display different replication/packaging efficiencies [18•].

The observation of reptarenavirus co-infection is unprecedented in arenavirus biology, as different superinfection exclusion mechanisms have been described for mammarenaviruses in cell culture [20]. *In vivo*, cross-protection between mammarenaviruses has also been documented, at least for closely related viruses [21–25]. It should, however, be kept in mind that reptarenaviruses have presently been detected only in captive snakes and it is thus unknown whether similar genotype dynamics occur in free-ranging reptiles. Indeed, breeding practices may have inadvertently created the ecological opportunity of multiple exposures eventually facilitating co-infections. In terms of gene sequences, mammarenaviruses and reptarenavirus show similarity both for the L and for the NP proteins, but no detectable homology for Z and GPC [6] (Figure 2a). Interestingly, Stenglein and co-workers detected sequence similarity between reptarenavirus (but not mammarenavirus) and filovirus glycoproteins [6]. Thus, either an ancient recombination event with a filovirus ancestor occurred at some point in arenavirus evolution or the ancestral arenavirus GPC was similar to that of filoviruses and subsequently diverged in mammarenaviruses [6]. We analyzed the hartmanivirus *GPC* gene using methods that detect remote homology and structural similarity. The best match for Haartman Institute snake virus (HISV) GPC, albeit limited to a short protein region, was with Junìn virus (JUNV, a mammarenavirus) glycoprotein (Figure 2b), whereas no detectable homology was found with filovirus sequences. This suggests that an ancient recombination event with a filovirus-like ancestor occurred on the lineage leading to reptarenaviruses. However, the evolutionary origin of arenavirus glycoproteins awaits clarification, as no significant homology with known proteins could be detected for the putative GPC sequence of fish arenaviruses, which show homologous regions with other arenaviruses for the L and NP proteins [2••] (Figure 2a).

Host associations

A general concept that emerged from large-scale meta-transcriptomic analyses is that, over long time frames, the associations between RNA viruses and their vertebrate hosts tend to be stable, despite frequent host-shifts [2••]. In the case of arenaviruses, though, long-term associations are difficult to establish, as the hartmanivirus *NP* and *L* genes seem to be more closely related to those of fish arenaviruses than to reptarenaviruses (Figure 1). Moreover, the natural hosts of reptarenaviruses and hartmaniviruses are presently unknown.

Over shorter time-scales, mammarenavirus-host associations were initially proposed to derive from co-speciation and co-dispersal [26,27]. The observation that most NW mammarenaviruses infect rodents from the subfamilies Sigmodontinae and Neotominae, whereas OW mammarenaviruses are preferentially found in rodents from the subfamily Murinae is in line with this possibility [27]. However, some mammarenaviruses have reservoirs in non-rodent mammals and host shifts were common both for NW and for OW mammarenaviruses [20,28]. In fact, two lines of evidence indicate that host-virus co-speciation did not play a major role in the observed associations between mammarenaviruses and their hosts. First, the divergence of the Murinae and Cricetidae families occurred around 20 million years ago (ya) [29], much earlier than the estimated divergence time of the OW and NW mammarenavirus lineages (at most 45 000 ya) [28]. Second, cophylogenetic reconciliation analyses indicated that co-divergence events do not represent the major determinant of mammarenavirus-host associations [28]. In fact, host geographic distribution rather than host relatedness most likely explains the preferential associations of mammarenavirus lineages with rodent subfamilies. Physical contact (either direct or through fomites) is necessary for a virus to be transmitted to a new host. Thus, the spatial overlap of the rodent subfamilies hosting mammarenaviruses may have represented an important factor for opportunistic host switches, as demonstrated in the case of two NW mammarenavirus clades [30] (see below for further discussion on geographic ranges). The observations that related host species often display overlapping spatial distribution and that viruses are more likely to switch successfully between closely related hosts [31] further support the idea that evolutionary events other than co-divergence contributed to shape the observed patterns of mammarenavirus-host associations.

Origin and geographic distribution

Phylogeographic analyses indicated that OW mammarenaviruses originated in Sub-Saharan Africa and NW mammarenaviruses in Latin America, areas that also harbor the largest diversity of mammarenavirus species [28] (Figure 3a). Wēnzhōu virus (WENV) and Loei River virus (LORV) are also likely to have an African origin and may have reached Asia in relatively recent times, possibly via human trade routes [28]. Similarly, anthropogenic factors are thought to have determined the wide distribution of the lymphocytic choriomeningitis virus (LCMV) (Figure 3a), due to its association with commensal rodents (*Mus musculus*) [28,32]. Notably, LCMV has rarely been reported in Africa (Figure 3a) and it was first isolated in this continent in 2015, in Gabon [33•]. Analysis of the viral sequences and of the rodent hosts indicated that LCMV was probably introduced recently in Gabon from America, together with its host (*M. musculus domesticus*). Indeed, analysis of all available LCMV genomic sequences suggested a North American origin for this virus [28]. However, because the origin of LCMV strains was dated ~6,742–700 ya [28,32] it remains to be explained how an ancestral African mammarenavirus reached the New World before human long-distance travel was developed.

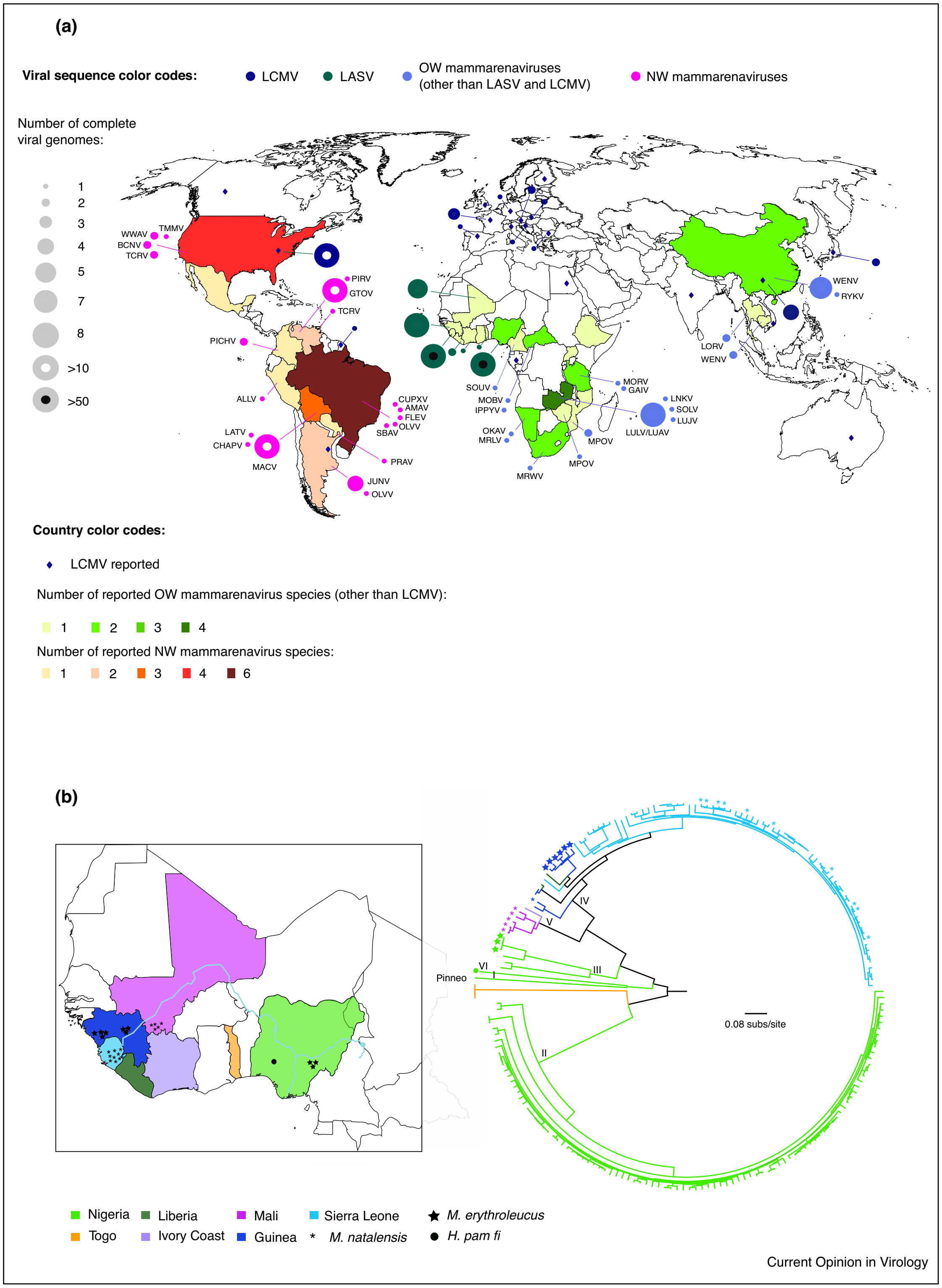
In general, several open questions remain concerning the ultimate origin of mammarenaviruses. If these viruses did not co-diverge with their hosts, how did they establish infection in African and American rodents? One possibility is that an ancestral arenavirus was independently transmitted to rodents (and other small mammals) in both continents from a common reservoir (fish, reptile or other). Alternatively, an ancestral arenavirus may have gained the ability to infect rodents in either Africa or America and may have spread to the other continent via an unknown host capable of long-range movements (e.g., a pinniped/cetacean, fish or bird).

It should, however, be kept in mind that evolutionary analyses of mammarenavirus origin and spread have been based on genomes with skewed geographic distribution (Figure 3a). Wider sampling of viral sequences both in terms of hosts and of geographic origin may unveil different scenarios. For instance, the recent description of the partial genome sequence of an unclassified mammarenavirus in the three-toed jerboa (Dipodinae subfamily) in China suggests an ancestral Asian range for some OW mammarenaviruses, as the jerboa virus falls basal to all OW mammarenaviruses [34].

Another open question relates to the fact that most mammarenaviruses seem to have relatively narrow geographic ranges: in several countries a single mammarenavirus species was reported (Figure 3a) and human hemorragic fevers tend to be confined to specific regions where the causative mammarenaviruses are also transmitted. For instance, Lassa fever has never been reported east of Nigeria, despite the widespread diffusion of its major host, the Natal multimammate mouse (*Mastomys natalensis*), throughout Sub-Saharan Africa [35]. Recently, Gryseels and coworkers reported that Gairo virus and Morogoro virus (GAIV and MORV, two OW mammarena viruses related to LASV, Figure 1) remain confined to a single *M. natalensis* subtaxon, despite the ecological opportunity to spread into the other subtaxon's range [36••]. The authors suggested that host-intrinsic barriers account for restricting mammarenavirus geographic ranges. Because different *M. natalensis* subtaxa are distributed east and west of Nigeria [35,36••,37], the diffusion of LASV may also depend on factors related to the distribution of rodent lineages. However, it is still unclear whether LASV is preferentially associated to specific *M. natalensis* subtaxa (i.e., mitochondrial lineages A–I and A–II) [36••,37]. Moreover, LASV was recently detected in rodents other than *M. natalensis*, such as *Hylomyscus pamfi* and *Mastomys erythroleucus* [38•], suggesting that it can bypass host barriers at the species level. In the case of *M. erythroleucus*, LASV sequences belonging to different lineages were detected (Figure 3b), suggesting either that the association with this rodent is long-standing or that the host shift from *M. natalensis* occurred at least twice. Therefore, biogeographical factors (e.g., major rivers) might substantially contribute to determine the diffusion of *Mastomys* rodents and, consequently, of LASV. An alternative possibility is that LASV has much wider diffusion than currently estimated, but Lassa fever is commonly unreported or misdiagnosed outside the endemic zone. Relevant to this is the identification of a distinct LASV lineage in multimammate mice in Mali (Figure 3b), a country where Lassa fever has rarely been reported [39]. It is presently unknown whether the Malian isolates are less transmissible to humans, tend to cause milder disease compared to other strains, or determine symptoms that make the diagnosis of Lassa fever more difficult [39]. The alternative possibility that LASV reached Mali only recently seems unlikely. Indeed, extensive genome sequencing in West Africa allowed a detailed analysis of LASV evolutionary history: the virus originated in Nigeria 1000–2000 ya and subsequently spread westward, reaching Mali and the Ivory Coast before arriving in Guinea and Sierra Leone [3••,40].

These large-scale analyses also clearly showed that LASV sequences tend to cluster based on broad geographic areas (Fig. LASV) and confirmed the notion whereby human infections are usually zoonotic [27], with limited role for human-to-human contact in infection spread [3••]. In Sierra Leone, where rodents have been actively sampled, human-derived and Mastomys-derived sequences are

Figure 3



Geographic distribution of mammarenaviruses.
(a) The number of distinct mammarenaviruses reported on a country basis is shown with color codes. Virus distribution was obtained via literature searches of papers reporting mammarenavirus identification by either genome sequencing or serology in distinct countries. Thus, white coloring and absence of LCMV reporting should not be interpreted as virus absence, but rather as missing information.
The number of available complete mammarenavirus genomes with their geographic origin is also shown (see legend). Information on genome

interspersed (Figure 3b), and sequences sampled over the same time-period do not necessarily cluster together [3••].

To date, the majority of human-derived LASV sequences belong to lineages II and IV (Figure 3b), possibly reflecting the fact that patients were recruited in specific geographic regions [3••]. However, the pattern is fast changing with the ongoing efforts to sequence viral genomes across Nigeria, which resulted in the detection of lineage III LASV in humans (see http://virological.org/t/2018-lasv-sequencing-continued/192/). The lack of human-derived LASV sequences belonging to the recently described lineage VI may instead indicate diversification of the virus in the alternative host *H. pamfi* (Figure 3b) and limited human exposure due to the non-commensal behavior of this rodent [38•].

Coding sequence evolution

Mammarenaviruses tend to cause persistent infections in their natural animal reservoirs, which contribute to viral maintenance and spread. Conversely, infection of non-natural hosts is often acute and severe (or even lethal) [41]. Thus, as for other RNA viruses, the host-range of mammarenaviruses may be potentially wide, but viral adaptation is likely required to turn a new host into a reservoir [42].

Extensive experiments *in vitro* have indicated that few amino acid substitutions in mammarenavirus proteins can drastically modulate the viral phenotype in terms of persistence and disease severity [43–52]. Although it is unclear whether these observations are translatable to viral dynamics in natural settings, adaptive changes in mammarenavirus coding sequences may accompany host-shifts and reservoir establishment.

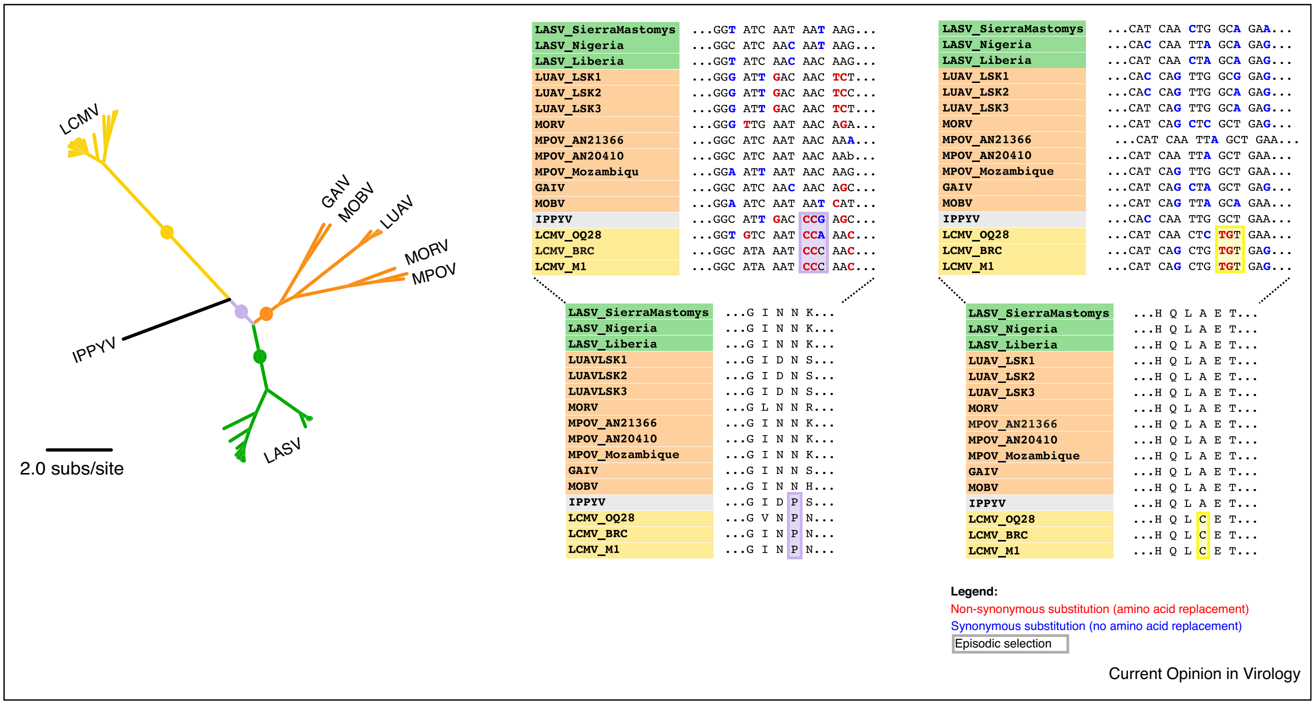
The evolution of coding sequences is commonly evaluated by comparing the substitution rates for non-synonymous (amino acid-replacing) and synonymous (non amino acid-replacing) substitutions (Figure 4). Because of structural or functional constraints, most proteins (both viral and non-viral) tolerate only minor sequence changes, resulting in fewer nonsynonymous substitutions than expected based on the synonymous substitution rate (negative or purifying selection). Indeed, the bulk of mammarenavirus and reptarenavirus coding sequences evolved under purifying selection, implying that most amino acid replacements reduce viral fitness [19,28,53•].

Recent studies however indicated that the speciation both of mammarenaviruses and of reptarenaviruses was accompanied by episodes of positive selection, a situation whereby amino acid replacements accumulate faster than expected on the basis of the synonymous substitution rate (Figure 4). In particular, the L protein was found to be the major target of positive selection [19,28,53•], suggesting that specific amino acid replacements in the mammarenavirus/reptarenavirus polymerases contribute to viral adaptation. The specific processes modulated by these changes remain nonetheless to be clarified. As indicated above, changes in L might tune persistence and virulence in novel hosts, eventually resulting in the establishments of new reservoirs. However, changes in other arenavirus proteins such as the glycoprotein were also shown to modulate viral persistence [46,49,54], but GPC was not found to have evolved under positive selection [28,53•]. An alternative possibility is that the selective pressures acting on the arenavirus L proteins is mediated by the host immune system. Recent evidence indicated that the LCMV L protein interacts with several innate immune molecules and the Mopeia virus (MPOV) polymerase can activate the RLR/MAVS pathway [55,56]. Nevertheless, the functional significance of such interactions in terms of evasion from immune surveillance remains to be assessed. Moreover, the viral nucleoprotein, which shows modest or no evidence of positive selection [28,53•], also interacts with several immune system components [57–60].

Notably, it has long been known that adaptive changes in the viral polymerase play an important role in the adaptation to mammalian hosts of avian influenza viruses [61]. Bird-infecting viruses encode polymerases with low activity in mammalian cells, where their replication is thought to be limited by insufficient synthesis of viral genomes [61]. During cross-species transmission, adaptive mutations in the viral polymerase enhance its activity and result in increased pathogenicity and transmissibility in mammals [61]. Reverse genetics experiments will be instrumental in determining whether positively selected changes in arenavirus polymerases also contribute to host shifts and in clarifying the viral phenotypes affected by the adaptive mutations. Of course, it will be of paramount importance to assess the role of adaptive changes in the modulation of disease severity in humans. Comparison of *L* genes from LASV sequences sampled in different geographic locations and isolated from different hosts indicated that positive selection also drove the evolution of the polymerase during the out-of-Nigeria migration of this virus [53•]. In particular, distinct selected sites were identified in rodent-derived and human-derived sequences, suggesting that amino acid replacements in LASV polymerase affect transmissibilty or disease severity in humans. An interesting possibility is that adaptive changes in the polymerase modulate its activity in a species-specific manner and confer the virus increased replication capability or faster propagation kinetics, eventually facilitating escape from immune surveillance in the

(Figure 3 Legend Continued) sequences was obtained from the NCBI database as of August 20th, 2018. Only viral species recognized by ICTV were considered. **(b)** Phylogenetic tree of complete LASV sequences sampled from either humans or rodents in West Africa. Color codes denote the geographic origin (see map and legend); symbols refer to the rodent host species (see legend).

Figure 4



Episodic positive selection in mammarenavirus *L* genes.
The comparison of the substitution rates for non-synonymous (amino acid-replacing, dN) and synonymous (non amino acid-replacing, dS) substitutions can be estimated to study the evolution of coding sequences. Under neutrality, these rates are expected to be equal and thus dN/dS = 1. Values of dN/dS lower than 1 indicate negative or purifying selection, whereas values higher than 1 are a hallmark of positive selection. Two sites that evolved under episodic positive selection in OW mammarenavirus *L* genes are shown as an example. The phylogenetic tree of *L* genes is drawn with dots denoting the branches of the phylogeny that were tested for evidence of selection (testing internal branches has the advantage that transient mutations on tip branches, which might represent deleterious transitory polymorphisms, do not affect selection inference). Nucleotide alignments of a few representative *L* genes are shown, with inferred synonymous and nonsynonymous substitutions marked in blue and red, respectively. Both in the nucleotide and in the amino acid alignments selected sites are shaded with the color matching the branch on which selection was inferred.

early stages of infection. Rapid viral proliferation of an LCMV strain was previously shown to associate with dysregulated adaptive immune responses in the initial stages of infection and with viral persistence [62].

Conclusions

Next-generation sequencing technologies have enormously increased our knowledge on arenavirus diversity in mammalian and non-mammalian hosts. These efforts have also made it clear that a large number of arenaviruses are likely to await discovery, as the range of sampled hosts and locations is still limited. In fact, wild reservoirs of reptarenaviruses/hartmaniviruses have still to be identified and the two antennaviruses are quite divergent, suggesting that fish harbor a large diversity of arenaviruses. Novel mammarenaviruses are also being described at increased pace. Future metagenomic/metatranscriptomic approaches will probably further expand the family and hopefully fill the gaps in our understanding of arenavirus origin and evolution.

More directly relevant to human health, knowledge about the spectrum and prevalence of arenavirus-associated disease is likely to be partial. Unknown arenaviruses may be pathogenic to humans and the disease-causing potential of known viruses is still incompletely understood (e.g., for WENV, [63•]). Metagenomics/metatranscriptomics of human samples will most likely be instrumental to address these issues, as well.

In general, a very important open question concerns the reason(s) why mammarenaviruses determine very different disease phenotypes in distinct mammalian hosts and the degree to which the viral and host genomes interplay in determining disease. The efforts to describe LASV diversity in West Africa have pioneered the use of viral genomics in the field of human infection disease. Despite these advances, it still remains unclear whether LASV lineages display differences in terms of human transmissibility and disease presentation/severity. It should also be noted that available human-derived LASV sequences are most likely biased, as they were obtained from hospitalized patients, whereas uncomplicated/mild cases remain un-diagnosed and un-sampled. This makes it difficult to identify the viral determinants (if any) that modulate human transmissibility and disease presentation. Future efforts aimed at diagnosing and sampling milder human cases might provide relevant insight.

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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.coviro.2018.11.001.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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The authors performed metatranscriptomic analysis on ∼220 invertebrate species in 9 metazoan phyla and discover more than 1400 novel viruses. Newly identify viruses largely expand viral diversity in metazoa. Hubei myriapoda virus 5 was described in this study.

2. •• Shi M, Lin XD, Chen X, Tian JH, Chen LJ, Li K, Wang W, Eden JS, Shen JJ, Liu L *et al.*: **The evolutionary history of vertebrate RNA viruses**. *Nature* 2018, **556**:197-202.

Using a large-scale metatranscriptomic approach the authors discover more that 200 novel viruses in fish, amphibians and reptiles. Data indicate that every family/genus of vertebrate-specific viruses known to infect mammals or birds is also present in cold-blooded vertebrates. The authors also show that, over long evolutionary times, RNA virus-host associations tend to be stable. The two antennaviruses were reported in this study (referred to as Wēnlǐng frogfish arenavirus 1 and Wēnlǐng frogfish arenavirus 2).

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